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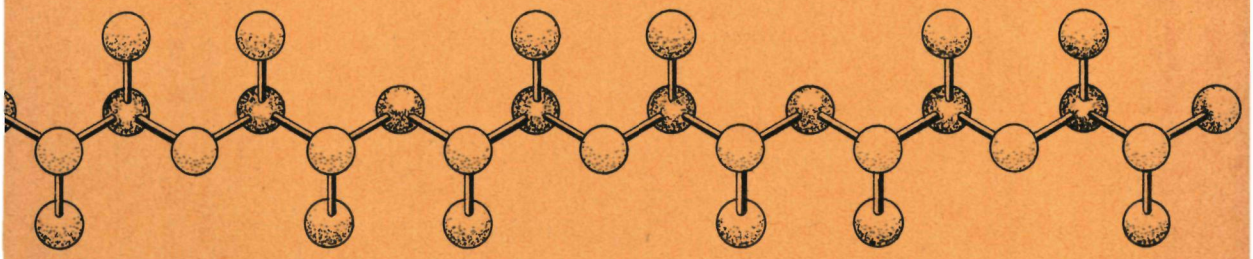
ESTIMATION OF FREE AND HEMOGLOBIN-BOUND CO_2



L.H.J. VAN KEMPEN

PROMOTOR

PROF. DR. F. J. A. KREUZER

ESTIMATION OF FREE AND HEMOGLOBIN-BOUND CO_2

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS DR. G. BRENNINKMEIJER,
HOOGLERAAR IN DE FACULTEIT DER SOCIALE WETENSCHAPPEN,
VOLGENS BESLUIT VAN DE SENAAT
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 8 JUNI 1972 TE 16 UUR

DOOR

LOUIS HENRI JOSEPH VAN KEMPEN

GEBOREN TE MAASTRICHT

NIJMEGEN 1972
DRUKKERIJ GEBR. JANSSEN N.V.

In commemoration of Professor Dr. F. J. W. Roughton, F. R. S., who followed this work with continued interest and valuable stimulation. His participation and advice are remembered with sincere gratitude. This great investigator who made decisive contributions to the physiology of blood gas transport during half a century, was not to see the completion of this thesis; he died suddenly on April 29, 1972, at the age of 72 years. The memory of this eminent scientist and the impact of his work will be with us in our future studies.

To the memory of my mother
thanking my father
for Marie Louise, Muriel and Anne-Marie.

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The Foundation 'De Drie Lichten', the 'Jan Dekker- en Dr. Ludgardine Bouwmanstichting' and the 'Dr. van Gils-Fonds' are gratefully acknowledged for their support in publishing this thesis.

Carbon dioxide transport in mammalian blood has been studied less thoroughly than oxygen transport. This may largely be due to the more complex nature of the process itself which involves interactions of CO_2 with water as well as with proteins (hemoglobin and possibly plasma proteins). Another reason is undoubtedly the more complicated methodology required to estimate CO_2 and its constituents in blood. Oxygen polarography has proven to be a reliable and fast method to determine oxygen partial pressure in liquids and spectrophotometry of hemoglobin provides an accurate means for estimating its fractional saturation with oxygen. In the case of carbon dioxide, however, equivalent methods are not available and the techniques used are, except for the Stow electrode, either indirect or unspecific. The gap of knowledge between oxygen and carbon dioxide transport is best illustrated historically by reference to the fact that the oxygen equilibrium curve of hemoglobin was established as early as 1870 whereas the first CO_2 equilibrium curve, which implies the combination of CO_2 with hemoglobin forming its carbamate compound (figure 4.8), has not been reported before 1971, i.e. at the same time when continuous recording of oxygen equilibrium curves in vivo (by combining fiber optic reflexion oximetry with oxygen polarography) is starting to be common practice in respiratory physiology.

The initial purpose of this thesis was to construct a fast-responding CO_2 microelectrode suited for in vivo application to physiological as well as clinical purposes. Results obtained with microelectrodes based on two different principles are reported in chapters 1, 2 and 3.

It was intended to study the kinetics of the combination of hemoglobin with CO_2 (carbamate formation) by these new electrodes in conjunction with a Hartridge-Roughton continuous flow apparatus. Such experiments were, however, postponed in view of the fact that equilibrium conditions of the CO_2-hemoglobin interaction appeared to be studied incompletely and interpreted incorrectly. Chapter 4 reports results obtained from experiments on the carbamate equilibrium of bovine hemoglobin at 37.0°C. The CO_2 binding sites

(terminal amino groups) of hemoglobin which were previously assumed to be equivalent in their CO_2 binding properties were found to differ significantly in their affinity for CO_2. A first approach to elucidate the affinity constants in terms of ionization and carbamate-equilibrium constants for individual binding sites is given in chapter 5 which deals with the preparation of bovine hemoglobin derivatives specifically modified at the terminal amino groups of either α or β chain. It is expected that carbamate studies on these derivatives will finally provide sufficient information to quantitatively elucidate the interaction of hemoglobin with CO_2.

All experimental work presented in this thesis was carried out in the Department of Physiology of the University of Nijmegen under the supervision of Prof. Dr. F. Kreuzer. The members of this department are gratefully acknowledged for their interest in the project and their cooperation throughout its course. The author would like to express his gratitude to Prof. Dr. L. Rossi-Bernardi (Cattedra di Enzimologia, University of Milan) who introduced him into the field of hemoglobin and made him familiar with some of the techniques used. He is indebted to Dr. John Kilmartin (M R C Laboratory of Molecular Biology, University of Cambridge) for providing detailed information about his procedure of preparing carbamylated horse hemoglobin derivatives. The valuable discussions with the late Ir. W. Reichert, Ir. T. Zelders, Ir. J. Spaan and Ir. P. Smulders as well as the mathematical advice of Drs. L. Hoofd are greatly appreciated.
The author is particularly indebted to Miss B. Ringnalda and the members of the gas laboratory for the numerous gas analyses performed, to Mrs. M. van de Ouderaa-Schram for competent help in preparing the modified hemoglobin derivatives, to Miss T. Hendriksen for providing current literature, to Mr. P. Breepoel, H. Deurenberg, R. Overkamp and P. Rottier for assistance in part of the experimental work, to Mr. K. Peters, W. Nagelvoort, F. Rombout, H. Smit, J. van Wamel and L. Ramakers for manufacturing the electrodes, to Dr. S. de Bruin and Dr. L. Janssen for providing their titration equipment. Thanks are due to Miss E. Maassen for carefully typing the manuscript, Mr. C. Nicolasen for preparing the figures and designing the cover, and Mr. A. Reijnen for photographical reproduction. The Consiglio Nazionale delle Ricerche (C N R, Rome) on behalf of the Dutch Organization for Basic Scientific Research (Z W O) is gratefully acknowledged for a research fellowship at the University of Milan during the initial stage of this project.

Chapter 1

THE CO_2-QUINHYDRONE ELECTRODE, A NEW METHOD TO MEASURE PARTIAL CO_2 PRESSURE IN GASES AND LIQUIDS

INTRODUCTION

Continuous *in vivo* recording of CO_2 pressure in the gas phase has long been common but continuous *in vivo* monitoring in fluids (particularly in blood) has been impeded by the relatively large dimensions and the slow response of the available devices. A method of measuring CO_2 pressure in liquids accurately and rapidly by a miniaturized instrument (analogous to the oxygen electrodes developed by Schuler and Kreuzer (1967) and Kimmich and Kreuzer (1969)) might presumably help to solve many problems in physiology and reaction kinetics as well as in clinical medicine.

The CO_2 electrode developed by Stow and Randall (1954), Stow, Baer and Randall (1957), and Gertz and Loeschcke (1958), has an accuracy of approximately 2 % at a Pco_2 of 50 mm Hg and a response time of 1.25 min for 99.5 % deflection (Pco_2 changing from 47 to 26 mm Hg) (Lunn and Mapleson, 1963). Microelectrodes as described by several authors (Constantine, Craw and Forster, 1965; Rybak and Penfornis, 1969) have a decreased response time but less stability and a short life time.

It was attempted first to improve the performance of this type of microelectrode applying the technological possibilities available at the time of the present study but no substantial progress could be achieved. Therefore another approach for measuring CO_2 pressure in fluids was followed with particular reference to miniaturization for *in vivo* monitoring. The quinhydrone electrode system, previously applied to pH measurements, was modified in such a way as to make it suited for determination of CO_2 pressure.

PRINCIPLE

Reversible oxidation-reduction systems in which hydrogen ions participate may

be suited for the determination of pH. For this purpose the oxidation-reduction potential should be a well defined function of the pH of the solution. It will be shown that the quinhydrone system satisfies this requirement to a high extent. Equations (1) and (2) describe the oxidation-reduction reaction and the corresponding electrode potential:

$$Q + 2H^+ + 2e^- \rightleftharpoons H_2Q \qquad (1)^1$$

$$E = E_o + \frac{RT}{2F} \ln \frac{a_Q (a_{H^+})^2}{a_{H2Q}} = E_o + \frac{RT}{2F} \ln \frac{a_Q}{a_{H2Q}} + \frac{RT}{F} \ln a_{H^+} \qquad (2)$$

a_Q, a_{H2Q}, a_{H^+} represent the quinone, hydroquinone and hydrogen ion activity, respectively, and E_o is the standard electrode potential.

If the activities of quinone and hydroquinone are the same the electrode potential becomes independent of any of these species and is a function of the hydrogen ion activity only. This condition may be fulfilled, according to Bates (1954), when using quinhydrone, an equimolar compound of quinone and hydroquinone.

This system has been introduced already in the pre-glass electrode years by Haber and Russ (1904) as a standard method of pH determination; E_o at 25.0°C was determined as 0.69992 V (Harned and Wright, 1933) or 0.69961 V (Hovorka and Dearing, 1935).

In a series of experiments the electrochemical properties of this system were reinvestigated with special emphasis on the influence of time, presence of oxygen, chloride concentration and light possibly affecting the electrode potential. Subsequently a membrane-covered electrode was constructed with as reference either a calomel or an Ag-AgCl electrode. The performance of this electrode was compared with that of the Stow-type electrode.

MATERIALS AND METHODS

Experiments were performed in an all-glass thermostated cell filled with a quinhydrone solution (usually 10^{-3} M/l). Quinhydrone was obtained from British Drug House and used without further purification. All stock solutions were stored in the dark.

Platinum wires were melted in glass. Physically pure platinum was used (ob-

[1] According to European convention (IUPAC Stockholm 1953).

tained from Drijfhout). The electrodes were cleaned electrolytically (voltage 10 V during 1.5 min; platinum electrode negative) in preheated Krause solution (15 g % Na_2CO_3, 5 g % KCl, 5 g % KCN and 1 g % NaCl in water), immersed in concentrated nitric acid and washed ultrasonically in bidistilled water.

As a reference electrode either a calomel or an Ag-AgCl electrode was used. The calomel electrode (Radiometer type K 401) filled with 0.1 N KCl or saturated KCl was placed in a side arm of the cell separated from the main solution by a sintered glass filter in order to prevent sticking of gas bubbles at the calomel liquid junction. A platinum or silver wire, melted in glass, was cleaned as described before, plated electrolytically with silver in a $KAg(CN)_2$ solution of 10 g/l with a current of 0.25 mA per cm^2 during 20 h, rinsed with bidistilled water and chlorided in 0.1 N HCl with 2.5 mA per cm^2 during 30 min. The electrodes were stored in 10^{-4} M/l KCl. Before each experiment the electrode was checked against a saturated calomel electrode in a solution of 12.82 g/100 ml KCl. The resulting potential should be between 18 and 24 mV in the temperature range of 20–30°C.

The quinhydrone solution was equilibrated by bubbling different preheated and humidified gas mixtures through the solution. pH was measured with a glass electrode (Radiometer G 202 C) connected to a Radiometer pH 4 meter. E values were measured by a Hewlett Packard voltmeter with digital read-out (HP 3439 A with 3244 A DC multi function unit). Quinhydrone, quinone and hydroquinone spectra were scanned by a double beam recording spectrophotometer (Hitachi Perkin Elmer model 124).

Construction of membrane-covered electrodes

The platinum calomel unit (Figure 1.1)

As shown in figure 1.1 a platinum ring (o.d. 4.9, i.d. 4.5 mm) was mounted around the liquid junction of a calomel electrode (Radiometer type K 401). Before mounting the ring was insulated by dipping it four times into 985 E Araldit (Ciba) during 15 min and drying it at 120°C. Then it was fixed to the calomel electrode by 820 Araldit. After drying it for seven days the surface of the ring was carefully polished to remove the insulation. Extreme care was taken not to damage the insulation at the edges of the ring. The combined electrode was mounted in a lucite housing to which a CO_2 permeable membrane was attached by an O ring. The whole system including a sample chamber was maintained at constant temperature by a thermostating cuvette.

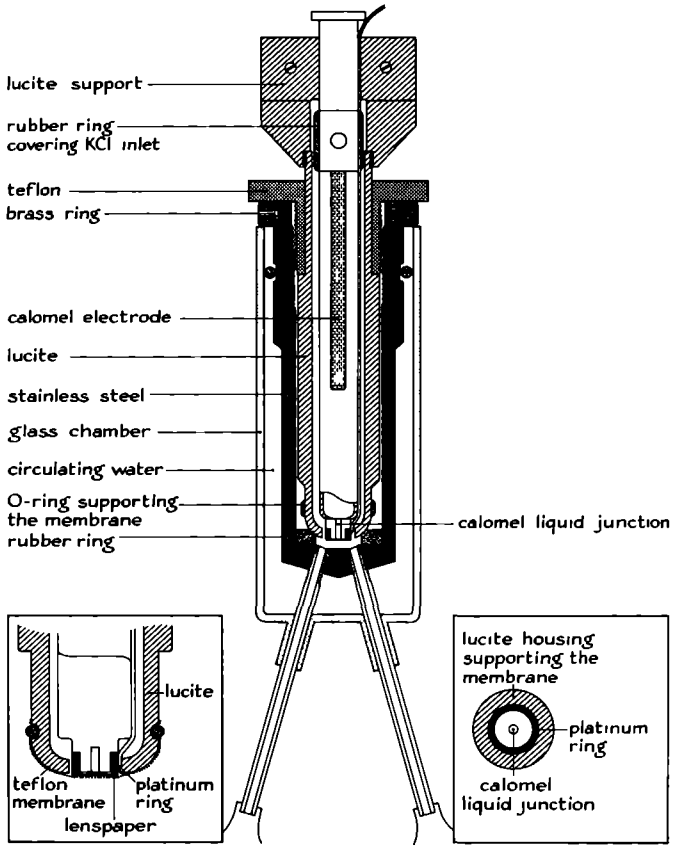


FIGURE 1.1 Construction of the CO_2-quinhydrone electrode

The platinum Ag-AgCl unit

A platinum plate with a surface of about 8 mm² together with a silver ring (o.d. 6.8, i.d. 5.8 mm) was melted into glass (not shown by figure). After the cleaning procedure the silver ring was chlorided as described above. The electrode was assembled and mounted in the same thermostating cuvette as the platinum calomel unit.

Operation of the electrode

The combined electrode was rinsed with the solution to be studied. A membrane (Silastic 25 μ or teflon 6 μ) was mounted and the electrode housing was filled

with a fresh solution of about 2 ml quinhydrone (10^{-3} M/l) and KCl (0.1 N) with or without sodium bicarbonate (10^{-3} M/l). The electrode unit was screwed into the lucite housing until the membrane was touched. Bulging of the membrane was avoided. A spacer (lens paper) was used in order to stabilize the fluid layer between membrane and electrode. The assembled electrode was screwed into the thermostating cuvette and allowed to stand for about two hours until the electrode potentials were stable (drift of 0.2 mV/h or less). A calibration curve with different CO_2 gas mixtures saturated with water vapor at electrode temperature was taken by plotting the electrode potential against the logarithm of the CO_2 partial pressure.

RESULTS

1. *Titration curve of quinhydrone*

A quinhydrone solution was titrated with KOH under nitrogen at 25.0° C. KOH was used because of its low alkaline error on the glass electrode. The pK value

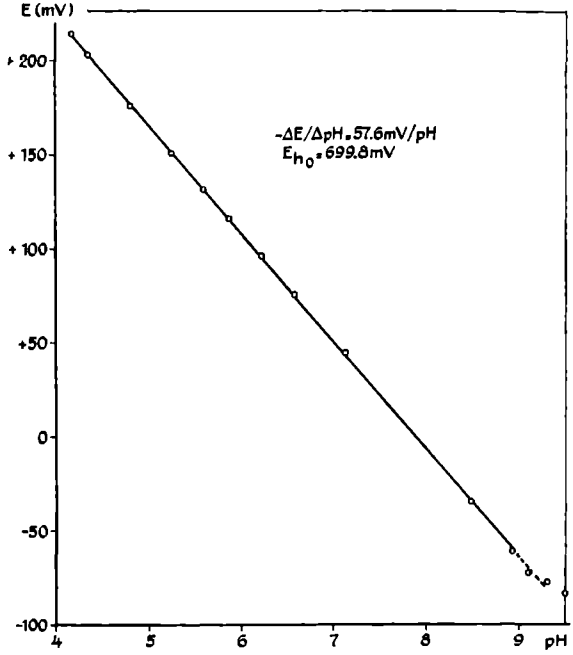


FIGURE 1.2 E *vs* pH curve of 10^{-3} M/l quinhydrone in water against a saturated calomel electrode at 25.0° C.

of hydroquinone was found to be 10.32 ± 0.08 (average of four determinations) in agreement with values reported in the literature (Weast and Selby, Handbook of Chemistry and Physics, 1966: 10.35 at 20.0°C).

2. *E vs pH curves in absence and presence of CO_2*

A quinhydrone solution was titrated with acid and alkali at 25.0°C. Figure 1.2 presents a typical E *vs* pH curve; in this case a value of $-\Delta E/\Delta pH$ of 57.6 mV/pH and a standard potential (E_o) of 699.8 mV were obtained. At pH values exceeding 9.2 a deviation from linearity was observed, resulting in a decrease of slope, partially due to ionization of hydroquinone and to autoxidation of hydroquinone and quinone. A $-\Delta E/\Delta pH$ value of 58.2 ± 2.7 mV/pH and an E_o value of 700 ± 4 mV were derived from 25 E *vs* pH curves with quinhydrone concentrations ranging from 10^{-3} to 25.10^{-3} M/l at 25.0 ± 0.1°C. Titration of the quinhydrone solution was also performed by equilibrating the solution with gas mixtures of different CO_2 concentrations. The E *vs* pH curve obtained in

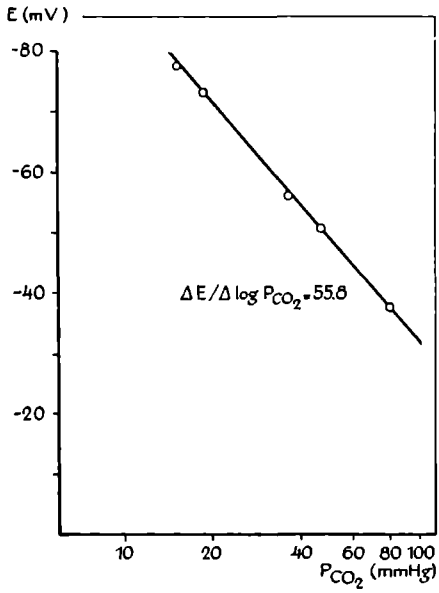


FIGURE 1.3 E *vs* log P_{CO2} curve of 10^{-3} M/l quinhydrone in 0.1 N KCl against an Ag-AgCl electrode at 25.0°C; 10^{-3} M/l $NaHCO_3$ was added to the quinhydrone solution.

this way did not differ significantly from the curve shown in figure 1.2, neither in slope nor in E_o.

Since bicarbonate increases the change in pH upon equilibration with gas mixtures of different CO_2 concentration, E *vs* pH and E *vs* log Pco_2 curves were taken after addition of sodium bicarbonate (10^{-3} M/l) to the quinhydrone solution (Figure 1.3). The presence of bicarbonate did not influence the slope of the E *vs* pH curve. Apart from the sign, the slope of the E *vs* log Pco_2 curve was found to be nearly equal to that of the E *vs* pH curve (55.8 and 58.2 resp.) resulting in a $-\Delta pH/\Delta \log Pco_2$ value of 0.96 in good agreement with experimental values obtained by the Stow electrode (Severinghaus and Bradley, 1958). In these conditions the redox system was therefore as sensitive to changes in CO_2 pressure as the glass electrode.

3. *Influence of chloride concentration*

When using an Ag-AgCl electrode as a reference electrode, relatively high chloride concentrations with respect to quinhydrone will be present in the electrode solution, which might disturb the quinone-hydroquinone activity ratio and therefore the electrode potential. Moreover quinone is known to be chlorinated especially at acid pH in the presence of suitable metallic catalysts.

Solutions of 10^{-3} M/l quinhydrone in water, 0.1 N KCl, 0.5 N KCl and 1 N KCl were titrated at constant temperature (24.9 $\pm$ 0.1°C) with acid and alkali; the pH and the corresponding electrode potential were measured against a saturated calomel electrode. From the resulting E *vs* pH curves no significant alteration in slope and position could be detected up to a chloride concentration of 0.5 N KCl. At 1 N KCl the curve was shifted to the right (change of E_o); furthermore there was a loss of stability and an increased scatter of the experimental values.

4. *Influence of oxygen*

Since a CO_2 electrode as suggested here is operating by a change in the oxidation-reduction state of the quinone-hydroquinone system according to variations in pH, it will only reflect partial CO_2 pressure accurately if its oxidation-reduction potential is not affected by oxygen as well.

After addition of acid or alkali a 10^{-3} M/l solution of quinhydrone was equilibrated during 20 min first with nitrogen, then with 11.4 % oxygen and eventually

again with nitrogen, the pH and electrode potential being determined after each equilibration. There was no significant difference between E *vs* pH regression lines in the presence or absence of 11.4 % oxygen at pH values up to about 7.5 (Figure 1.4)

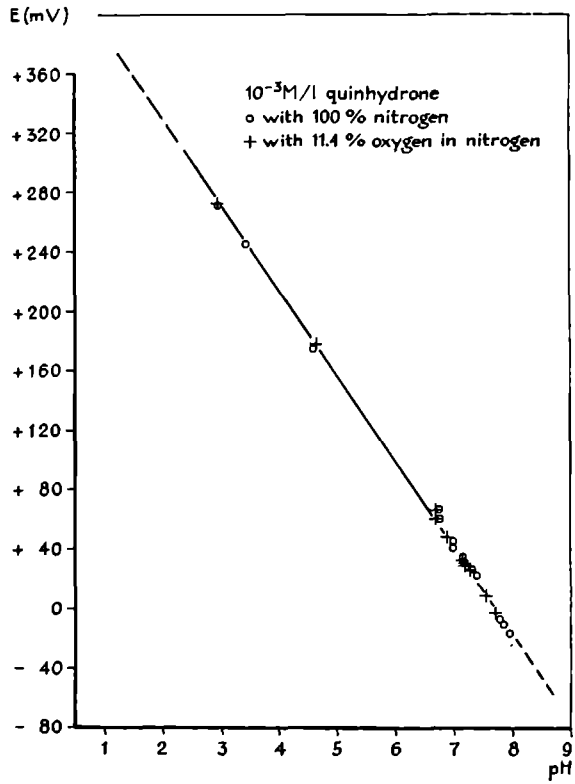


FIGURE 1.4 E *vs* pH curve of 10^{-3} M/l quinhydrone in water with and without oxygen (11.4 %).

5. *The influence of time and light on the spectral properties of quinone, hydroquinone and quinhydrone solutions in water and in* 0.1 *N KCl*

Stock solutions of quinone, hydroquinone and quinhydrone, all 10^{-3} M/l in water or 0.1 N KCl, were divided into two parts, one stored in the dark and the other exposed to daylight and at night to a 100 W bulb at a distance of about 40 cm. Visible and ultraviolet (U.V.) spectra of these 12 solutions were taken after 6, 22, 46 and 70 h. The pH of each solution was measured at the same

time. The influence of bicarbonate on these spectra was studied similarly by adding 10^{-3} M/l $NaHCO_3$ to freshly prepared stock solutions described above. All solutions were left in contact with air.

Visible spectra

Quinone and hydroquinone do not show much absorption in the visible spectrum contrary to the U.V. region. There is some minor absorption at 430 mμ, but the molar extinction coefficient (ε) of quinone is only about 20 and that of hydroquinone around 0.1. Quinhydrone, however, and other compounds of equivalent resonance structure present a very strong absorption of the order of 10^4–10^5 (Michaelis and Granick, 1944). Therefore an increase of optical density (O.D.) at 430 mμ may be attributed exclusively to the presence of quinhydrone. A native mixture of quinone and hydroquinone, containing only small amounts of quinhydrone, should have low optical absorption. Figure 1.5 a however indicates that the O.D. becomes and remains higher soon after continued exposure to light. The concomitant decrease of pH suggests that this change in O.D. may be due to the formation of acid reaction products. In contact with air hydroquinone and quinone are known to be oxidized, forming brown acidic polymeric products (Eller and Koch, 1920), the rate of formation depending on the pH of the solution. Since the solutions are prepared in the

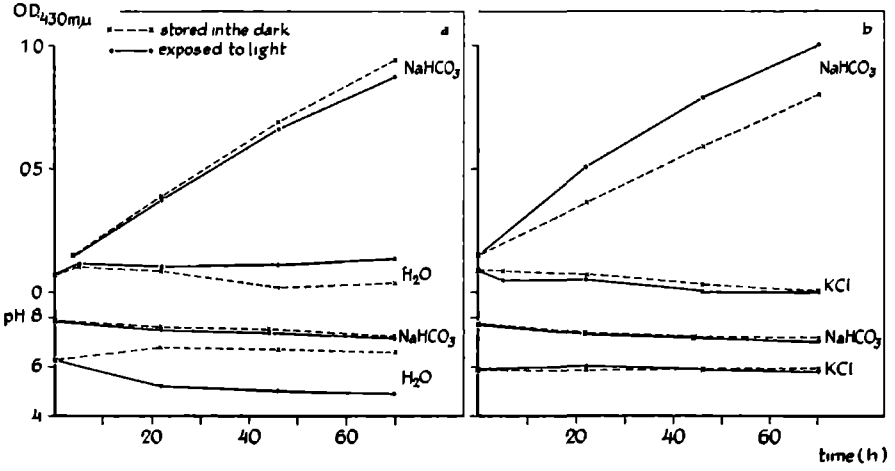


FIGURE 1.5 Influence of time and light on the optical density at 430 mμ and on the pH of quinhydrone solutions with or without 10^{-3} M/l $NaHCO_3$:
a 10^{-3} M/l quinhydrone in water;
b 10^{-3} M/l quinhydrone in 0.1 N KCl.

presence of light, these changes presumably occur already at this moment. When the solution is stored in the dark after having been prepared, the O.D. becomes very low with time and the pH remains higher (Figure 1.5 a) which may indicate that the changes induced by light are reversed.

According to La Mer and Rideal (1924) and Dingemans (1928) the velocity of autoxidation and therefore the formation of acid polymer compounds increases with rising pH as seen from the course of the O.D. after adding 10^{-3} M/l bicarbonate. Here the decrease of pH is more moderate due to the increased buffer capacity of the bicarbonate solution. There is practically no effect of light on the change in O.D. here.

Data obtained from quinhydrone solutions in 0.1 N KCl are plotted in figure 1.5 b. The O.D. decreases with time whereas the pH remains constant. This excludes the possibility of formation of acid polymerization products. There is practically no influence of light. Addition of bicarbonate accelerates the formation of quinhydrone-like acid products as seen also from the decreasing pH. Light enhances this process.

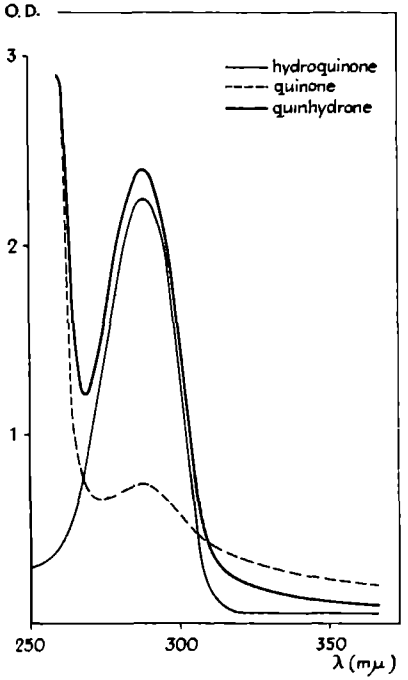


FIGURE 1.6 U.V. spectra of quinone, hydroquinone and quinhydrone in water.

Figure 1.6 demonstrates that in water, apart from a high intensity maximum near 247 mμ, quinone shows in the U.V. region a band of moderate intensity near 292 (ε∼500), the wave length position and degree of absorption varying with solvent composition (Scheibe, 1926; Braude, 1945). Hydroquinone spectra have an absorption maximum at about 290 mμ and a relative minimum at 250 mμ (Michaelis and Granick, 1944).

It is concluded from the absence of any shift of the absorption maximum and of any change in the extinction coefficient between these spectra in water and in 0.1 N KCl, that no substitution of chloride has occurred in these conditons as suggested though never proved by several authors (La Mer and Baker, 1922; Harned and Wright, 1933).

In figures 1.7 a, b and c the spectra of quinhydrone in water, in 0.1 N KCl and with bicarbonate in 0.1 N KCl after varying periods of time are presented when

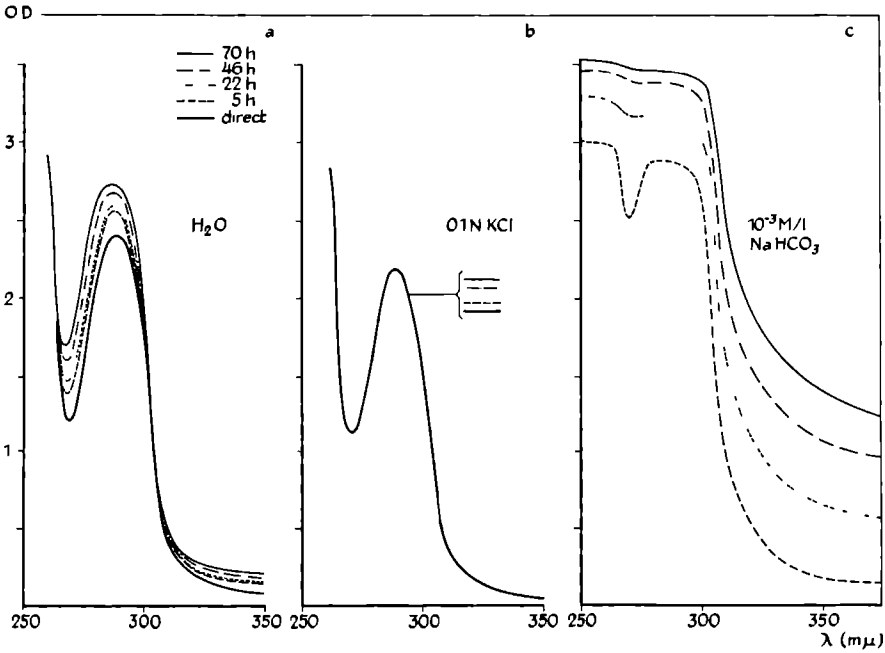


FIGURE 1.7 U V. spectra of quinhydrone solutions exposed to light for 0, 5, 22, 46 and 70 h.

a quinhydrone in water;

b quinhydrone in 0.1 N KCl;

c quinhydrone in 0.1 N KCl with 10^{-3} M/l $NaHCO_3$.

exposed to light. The spectral changes of quinhydrone in water may be largely attributed to the formation of oxidation products of quinone (Figure 1.7 a). This process evolves considerably more slowly in the absence of light (not shown here); no spectral changes were observed after storage of quinhydrone in the dark for 22 h. Spectra of hydroquinone in water undergo changes in the presence and absence of light which suggests a reversible oxidation to quinone. The spectral changes of quinhydrone solutions as observed in water are completely absent in 0.1 N KCl (Figure 1.7 b). The same holds for quinone with or without light. Hydroquinone, however, shows reversible changes related to quinone formation which are not influenced by light.

Considerable changes (increase of absorption in the U.V. region) in the spectra of quinhydrone (Figure 1.7 c), quinone, and hydroquinone in water or in 0.1 N KCl were seen immediately after adding 10^{-3} M/l $NaHCO_3$. These alterations are highly suggestive of quinone oxidation and polymer formation. No specific effect of light was noticed.

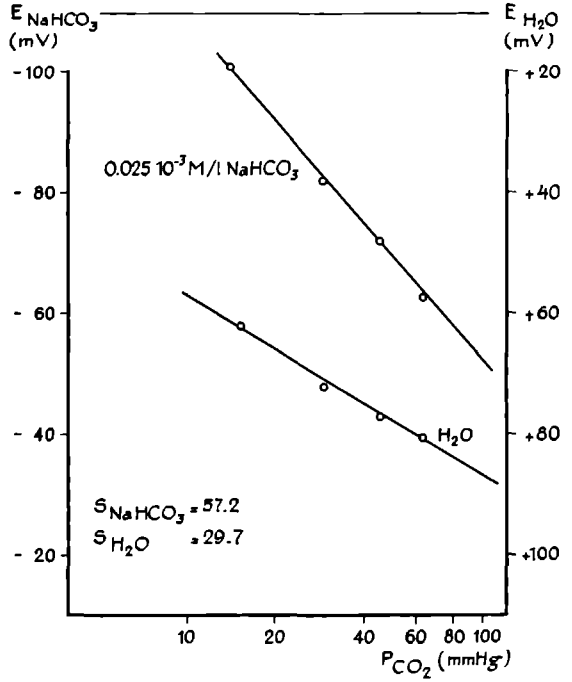


FIGURE 1.8 Calibration lines of the CO_2-quinhydrone electrode with bovine blood equilibrated with various CO_2 concentrations at 37.0°C with or without bicarbonate added to electrode solution.

Stability and calibration

The stability of the electrode with 10^{-3} M/l quinhydrone in 0.1 N KCl is better than 0.2 mV per hour which is equal to the drift of the CO_2 electrode (Radiometer type E 5036) according to Stow *et al.* (1957). Upon addition of bicarbonate the stability is somewhat less. Repeated calibration within short intervals of time is therefore required. Calibration lines with CO_2 gas mixtures were similar to that shown in figure 1.2. Figure 1.8 was obtained with bovine blood equilibrated with CO_2 at 37.0°C with and without bicarbonate added to the electrode solution. In both cases the values of the sensitivity ($-\Delta pH/\Delta \log Pco_2$) agreed with corresponding values found with the Stow electrode (Severinghaus and Bradley, 1958).

Response time and error

The response time for 95 % deflection at 35.6°C is shown in figure 1.9 and equals 120 sec (on) and 180 sec (off) after a Pco_2 step change between 5.5 and

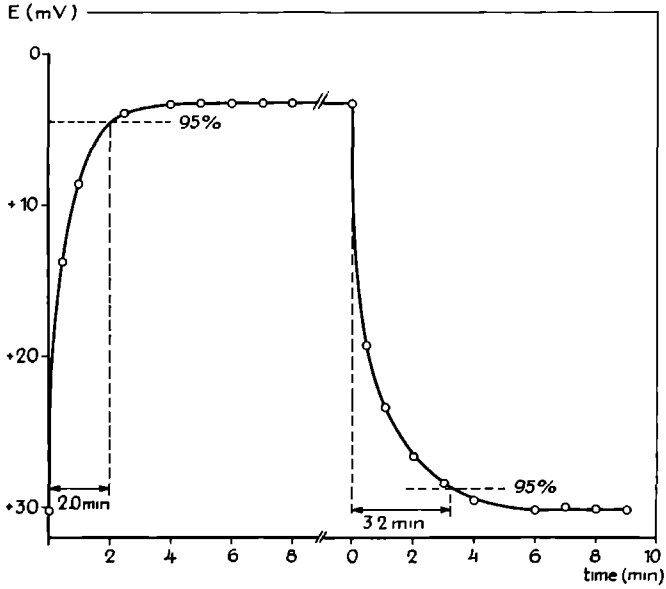


FIGURE 1.9 Response time of the CO_2-quinhydrone electrode after a ten-fold increase of P_{CO_2} (from 5.5 to 51.8 mm Hg). Electrode with 6 μ teflon membrane; no bicarbonate added to the quinhydrone solution.

51.8 mm Hg (6 μ teflon membrane, no bicarbonate added to the quinhydrone solution). This response time is comparable to that obtained with the Stow electrode (Lunn and Mapleson, 1963).
The standard deviation estimated from 12 determinations at a Pco_2 of 42 mm Hg without bicarbonate added to the quinhydrone solution is about twice as high (4 %) as that in the Stow electrode.
Similar results were obtained when using an electrode system with an Ag-AgCl electrode as a reference.

DISCUSSION

The quinhydrone electrode, widely applied before the introduction of the glass electrode and still in use even in recent times (Dahlgren and Goodfriend, 1970), has proved to be a good substitute for the hydrogen electrode. However, its performance in practice is subject to several limitations, particularly at the extremes of the pH scale, the main limitations arising from effects disturbing the quinone-hydroquinone activity ratio. Some of these will be discussed as far as they might interfere with Pco_2 estimation.

1. *Acid-base reactions*

The acid-base reactions of hydroquinone and quinone may affect this ratio to a certain extent depending on the pH of the solution. At alkaline pH the concentration of undissociated hydroquinone is lowered according to its dissociation constant, resulting in an increase of E; at very low pH E is expected to be diminished because of the alkaline properties of quinone in strongly acid solution. These influences occur with hydroquinone ($pK = 10.32$) at pH values higher than 8 and with quinone below a pH of 2 (Biederman, 1956); these regions are outside the working range of the quinhydrone electrode used for CO_2 determination.

2. *Semiquinone formation*

It was suggested by Michaelis (1935) that in many organic bivalent oxidation-reduction transitions, stable free radicals (semiquinones) were involved which had an oxidative state intermediate between reduced and oxidized form. The presence of semiquinones as an intermediate step in the reversible oxidation-reduction of quinhydrone would obviously interfere with reaction 1 (above) and

would change accordingly the slope of the E *vs* pH curve. Alterations in slope were demonstrated by Michaelis (1935) for many compounds, strongly supporting the existence of these intermediates; but such deviations are absent in the case of quinhydrone in the pH region being studied here. At acid and neutral pH, the concentration of semiquinone is extremely small because of its tendency to pass to quinone and hydroquinone or to their combined form of quinhydrone (Wagner and Grünewald, 1940). Therefore no inflections in the E *vs* pH curve are seen in this pH region.

3. *Autoxidation*

La Mer and Rideal (1924), Dingemans (1928), and James, Snell and Weissberger (1938) studied the autoxidation of hydroquinone by measuring its oxygen consumption at various pH values. It was found that at alkaline pH (8.2) the first products of this oxidation were quinone and hydrogen peroxide, the reaction velocity being proportional to the concentration of dissolved oxygen and of hydroquinone, and to the square of the hydroxyl ion concentration. At acid and neutral pH no oxidation in the presence of air could be detected. This is also confirmed by the observations shown in figure 1.4 on quinhydrone solutions equilibrated with 11.4 % oxygen during 20 min.

Quinone too is oxidized at alkaline pH. In contact with air oxidation starts slowly at a pH of about 7.4, but rapidly increases at higher values (Dingemans, 1928), forming oxyquinone which in turn polymerizes to dark brown acid products. From elementary analysis a composition of $(C_6H_4O_3)$ was obtained for these so-called humic acids (Eller and Koch, 1920) whose molecular weight and chemical structure are unknown at present.

The spectral changes observed in quinone and quinhydrone solutions (Figure 1.7) are similar to those found in the enzymatic oxidation of catechol where polymeric products are formed too (catechol melanin; Mason, 1949). It is generally assumed that in all these autoxidations free radical reactions are involved. Therefore both light by stimulating semiquinone formation (photodissociation of quinone; Leighton and Forbes, 1929) and alkaline conditions by stabilizing these anionic radicals will promote autoxidation. Chloride ions seem to offer some protection against this light effect.

From figures 1.5 and 1.7 c it is seen that the electrode solution never should be allowed to exceed pH values of about 7.2. Thus addition of about 10^{-3} M/l bicarbonate, although increasing the sensitivity considerably (up to 100 %),

should always be avoided in those cases where stable and accurate readings are required at low Pco_2 values.

4. *Influence of chloride*

Chemical reactivity of quinone

Instability of electrode potentials as sometimes observed in acid solutions containing a high concentration of chloride ions (2 N) is said to result from chlorination of quinone (La Mer and Baker, 1922; Harned and Wright, 1933; Hovorka and Dearing, 1935). At a chloride ion activity of 0.1 N KCl as applied in the electrode solution and commonly used in determinations of the quinhydrone standard potential, no chemical evidence could ever be presented for the formation of chlorohydroquinone.
In figure 1.7 no changes in U.V. spectra were observed compatible with chlorination of quinone (Braude, 1945). Therefore it seems rather unlikely that chlorination of quinone will seriously affect Pco_2 estimation.

Salt error

It has been demonstrated by Sørensen, (1923) and Linderstrøm-Lang (1927) that several ions have a disturbing effect upon the quinone-hydroquinone activity ratio. This so-called salt error appeared to be dependent on the nature and the activity of the ion involved and on pH and temperature of the solution. At low pH it is practically independent of pH.
From the data of Hovorka and Dearing (1935) the effect of 0.1 N KCl may be calculated to raise the measured pH value by 0.00372 at 25.0°C and pH of about 2. It was found that at pH values above 4 the salt error is considerably higher, especially in 1 N KCl solution, together with an increased scatter at high chloride activities. According to Gabbard (1947) this larger salt error at higher pH values may be due to the acid-base reaction of hydroquinone. It therefore seems necessary to hold chloride ion activity in the electrode solution at a constant level which is achieved by using a 0.1 N calomel electrode as a reference.

5. *Preparation and properties of the CO_2-quinhydrone electrode*

Choice of the metal

On the basis of rather conflicting evidence obtained by several authors (Biilmann

and Lund, 1921; Biilmann and Jensen, 1927; Rosenthal, Lorch and Hammett, 1937) both platinum and gold are recommended as the optimal material to be used in the construction of highly reproducible quinhydrone electrodes. In this investigation platinum was chosen according to the suggestion of Livingston *et al.* (1932) and Lammert *et al.* (1931), based on 22000 measurements in 7500 cells. Gold electrodes were less reproducible and sometimes showed inexplicably erratic behavior, but seemed to be less sensitive to the presence of air.

Stability and reproducibility

According to the original papers by Biilmann and Lund (1921), and Biilmann and Jensen (1927), quinhydrone electrode potentials, as measured between two different electrodes or against the hydrogen electrode, could be estimated with a precision of 0.05 mV or better. Stability is of the order of 0.02 mV during about 4 h according to these authors and reproducibility is said to be excellent (Veibel, 1923). It was pointed out (Livingston *et al.*, 1932) that these values refer only to optimum conditions and are highly dependent on the preparation of the electrode and the composition of the solution to be studied. Under circumstances as mentioned above it was never possible to get a stability better than 0.1 mV per hour, a deviation five times higher than in the experiments of Biilmann but low enough to give reliable pH values as compared with the glass electrode. The loss of stability is probably due to the construction of the platinum electrode which could not be cleaned as rigorously as recommended by Biilmann and Jensen (1927) and Livingston *et al.* (1932).

The properties of the quinhydrone electrode as CO_2 electrode are fully comparable to those of the Stow electrode. Stability and response time are of the same order of magnitude. The error in Pco_2 is somewhat higher which may be largely attributed to imperfections in the construction of the cell holder. Compared with the Stow electrode it has the advantage of being an inexpensive low impedance electrode which may be miniaturized more easily and therefore might be adapted to *in vivo* Pco_2 estimation.

SUMMARY

An electrode is described consisting of a combined platinum-calomel electrode (or Ag-AgCl electrode) immersed in 0.1 N KCl solution with 10^{-3} M/l quinhydrone and separated from the medium by a CO_2 permeable membrane. The

whole system is thermostated. The resulting electrode potential is linearly related to the logarithm of the CO_2 partial pressure of the medium.

From 25 E *vs* pH curves at 25.0 $\pm$ 0.1°C with quinhydrone concentrations ranging from 1 to 25 10^{-3} M/l a $-\Delta E/\Delta pH$ value of 58.2 $\pm$ 2.7 mV/pH was derived and E_0 was 700 $\pm$ 4 mV.

Addition of 0.25 M/l $NaHCO_3$ to the electrode fluid changed $\Delta E/\Delta \log Pco_2$ from 29.7 to 57.2. The effect of chloride ions and oxygen on the electrode potential and the influence of time and light on the spectral properties of quinhydrone were studied. Stability, response time, and error of Pco_2 in this electrode system were of the same order of magnitude as in the Stow electrode.

Chapter 2

A SINGLE-UNIT CO_2-OXYGEN SENSING MICROELECTRODE SYSTEM

INTRODUCTION

In this chapter a microelectrode system is presented based on the quinhydrone oxidation-reduction principle described previously. In addition to and independent from its CO_2 sensing properties this system is demonstrated to function as a Clark-type oxygen electrode as well.

MATERIALS AND METHODS

Preparation of platinum electrodes

The influence of shape and size of the platinum electrode on the slope of the E *vs* pH curve of quinhydrone was studied on thirteen platinum electrodes of three main forms (ring, plane and rod) and surface areas ranging from 0.003 to 23 mm^2. Nine of these electrodes with surface areas of 0.2 mm^2 or larger were prepared by melting physically pure platinum (obtained from Drijfhout) in glass. For the other four electrodes of lower surface area microelectrodes were used as described by Schuler and Kreuzer (1967) and Kimmich and Kreuzer (1969). All electrodes were treated as described previously.

E *vs* pH curves were obtained in a thermostated cell at 20.0 $\pm$ 0.2°C filled with a quinhydrone solution of 10^{-3} M/l in 0.1 N KCl by adding appropriate amounts of acid and alkali. E values of each platinum electrode were determined against a saturated calomel electrode by a Hewlett Packard voltmeter with digital read-out (HP 3439 A with a 3244 A DC multi function unit). pH was estimated simultaneously by a combined glass electrode (Radiometer G 202 C) connected to a Radiometer PH 4 pH meter.

Construction of the microelectrode system

The cylindrical probe with an external diameter of 3.5 mm and a length of

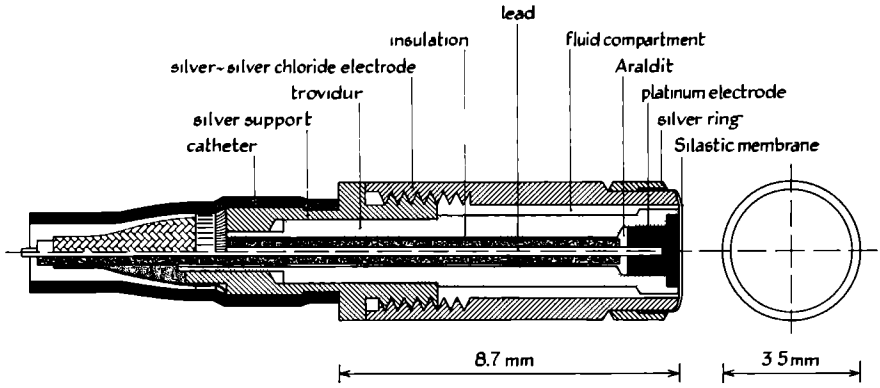


FIGURE 2.1 Construction of the CO_2-quinhydrone microelectrode system.

8.7 mm is shown in figure 2.1. It consists of a central platinum electrode with a surface area of about 2.5 mm^2 sealed with Araldit into a holder of polyvinylchloride (Trovidur) and surrounded by a tubular Ag-AgCl reference electrode. The tip of the probe is covered by a CO_2 permeable membrane (Silastic 25 μ or teflon 6 μ) fixed to the reference electrode by a silver ring.

A quinhydrone solution of 10^{-3} M/l in 0.1 N KCl is introduced into the probe after screwing off the ring-shaped reference electrode. Since an earlier prototype showed loss of the fluid layer between electrode and membrane after use for some hours a liquid reservoir has been added. The design of this electrode is similar to that of the oxygen electrode constructed by Schuler and Kreuzer (1967). Temperature control has been achieved by inserting the probe into a flow-through cuvette according to Kimmich and Kreuzer (1967). By heating the calibrating gases, which were sucked through the cuvette at a constant flow rate of about 6 ml per sec, this device maintained a constant temperature at the electrode tip to within 0.1°C for changes of ambient temperature between 0 and 30°C.

Gas mixtures were prepared by mixing pure gases in appropriate proportions in a gas mixing pump (Wösthoff type M 300/a).

RESULTS

1. *Influence of shape and size of the platinum electrode*

E *vs* pH curves were studied on thirteen platinum electrodes with three main

forms (ring, plane, rod) and surface areas ranging from 0.003 to 23 mm². Only those electrode dimensions were considered which might be important for the construction of microelectrodes. With each of these electrodes, prepared and cleaned in the same way, at least two E *vs* pH curves were taken. The results are shown in table 2.1. There is no dependency on surface area above 0.7 mm²

TABLE 2.1 Influence of surface area and shape of the platinum electrode on the slope of the E *vs* pH curve of quinhydrone at 25°C.

Number	Shape	Area mm²	Slope mV/pH	Standard deviation
1	Ring	0.003	39.9 (10.5*)	4.0
2	Ring	0 003	33.0 (6.5*)	4.2
3	Plane	0.07	54.8 (26.5*)	4.8
4	Plane	0.07	52.6 (30.3*)	5.4
5	Plane	0.2	57.0	2.7
6	Rod	0.5	55.9	2.0
7	Rod	0 7	58.1	2.2
8	Plane	3.1	60.4	1.5
9	Ring	5.9	58.2	1.5
10	Rod	6.7	57.1	2.1
11	Plane	7.1	60.0	1.6
12	Rod	10.4	60.9	0.9
13	Rod	23.3	60.4	0.8

* Discontinuity between pH 5 5 and 6.5 with subsequent decrease of slope.

whereas the slope is decreased and the curve deviates from linearity below this limit. No differences are observed between electrodes of different shape (see numbers 8–10). The variations in slope for a single electrode are 4 % of the mean value or less for numbers 7–13, but increase considerably up to about 10 % for the lower numbers. When platinizing the electrodes of lower surface area, the slope values rise to about 58 mV except for numbers 1 and 2.

2. *Properties of the microelectrode used as a CO_2 electrode*

E *vs* pH curves were studied in the membrane-covered electrode by filling it with quinhydrone (10^{-3} M/l) and KCl (0.1 N) dissolved in phosphate buffers of varying pH (ranging from 5.8 to 7.0). The probe was inserted into the cuvette thermostated at 37.0°C, and continuously flushed with CO_2 free nitrogen (or oxygen). Again a linear relationship was obtained with a $-\Delta E/\Delta pH$ value of 60.5 ± 1.6 mV/pH (standard error S.D. of 3 determinations). After some days

the slope values tended to decline (to 54.8, 51.7, 52.7 mV/pH) but could be restored completely (58.8 ± 1.2 mV/pH, 3 determinations) after platinizing the central platinum wire (1 % platinum chloride and 0.02 % lead acetate in 0.025 N HCl, 200 mA cm^{-2} for 2 min).

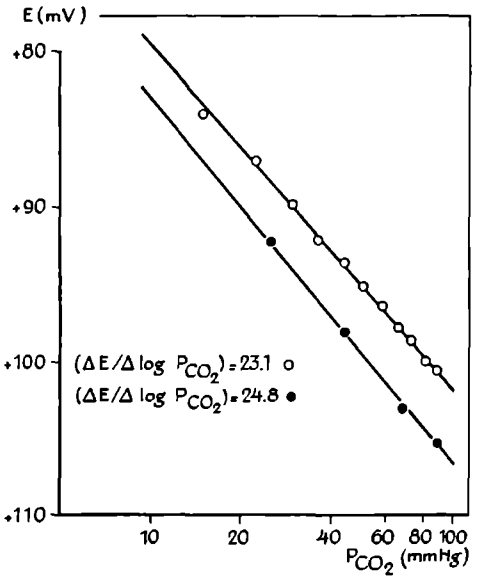


FIGURE 2.2 E *vs* log P_{CO_2} lines for CO_2 probes with blank (o) and platinized (•) platinum electrodes.

Calibration was carried out with gases of various CO_2 concentrations. Figure 2.2 shows calibration lines for probes with blank and platinized electrodes, both filled with 10^{-3} M/l quinhydrone in 0.1 N KCl. The $\Delta E/\Delta \log Pco_2$ values of 23.1 (blank) and 24.8 mV mm Hg (platinized) are somewhat lower than in the macroelectrode, thus giving sensitivity values ($-\Delta pH/\Delta \log Pco_2$; Severinghaus and Bradley, 1958) of about 0.42.

The 95 % response time of the probe to a step change in Pco_2 (between 24.9 and 88.9 mm Hg), with a blank platinum electrode covered by 6 μ teflon, is about 50 sec (on) and 65 sec (off). The stability of this microelectrode system is considerably inferior to that of the macroelectrode described in the previous chapter. There is a drift of at least 10 mV per hour for blank and of about 4 mV for platinized electrodes, usually but not always in acid direction. Drifting was

not reduced by stabilizing the fluid layer with a spacer (Joseph paper). The Ag-AgCl reference electrode tested against a saturated calomel electrode in 12.83 g % KCl was found to be constant within 0.1 mV per hour.

3. *Properties of the microelectrode used as an oxygen electrode*

The oxygen polarogram was studied in the CO_2 probe as well as in a similar probe with a central platinum electrode of lower surface area (0.07 mm², table 2.1, no 3), both covered by 6 μ teflon. Polarograms and calibration lines were taken from both electrodes filled alternately with 10^{-3} M/l quinhydrone in 0.1 N KCl, 10^{-3} M/l quinhydrone and 10^{-3} M/l $KHCO_3$ in 0.1 N KCl, and 0.1 N KCl only. In all cases a polarogram with a broad horizontal plateau of at least 500 mV and a calibration line passing through the origin and linear up to 100 % oxygen were obtained. In figure 2.3 polarogram and calibration

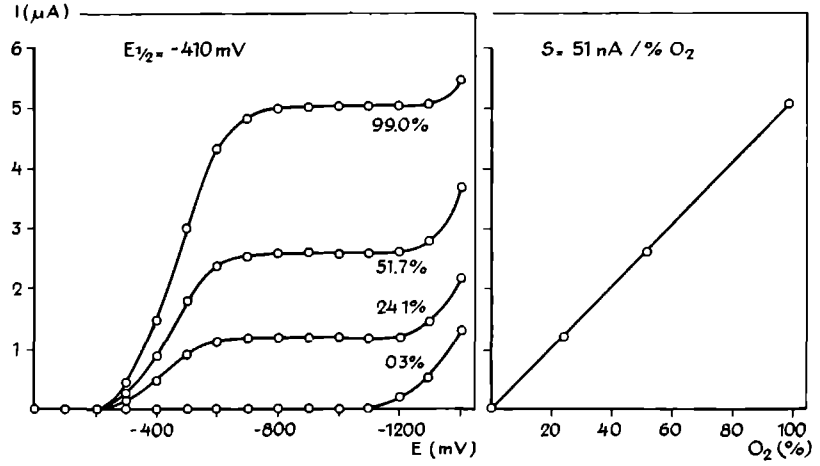


FIGURE 2.3 Oxygen polarogram and oxygen calibration line for a probe with a cathode surface area of 0.07 mm². Electrolyte composition is 10^{-3} M/l quinhydrone in 0.1 N KCl. The probe is covered by 6 μ teflon.

line are shown for the electrode with the smaller surface area (cathode) filled with 10^{-3} M/l quinhydrone in 0.1 N KCl. Half wave potential ($E_{\frac{1}{2}}$) is —410 mV and sensitivity ($\Delta I/\Delta \%O_2$) is 51 nA/$\%O_2$. Similar values were obtained when bicarbonate was added or 0.1 N KCl was used only. Residual current, i.e. current in the absence of any oxygen, was always less than 0.1 μA. The polaro-

gram of the probe with the larger cathode area was essentially the same as that shown in figure 2.3 but the current output was about 15 times as high. Again this polarogram was not affected by addition of quinhydrone with or without bicarbonate. Response time for 95 % deflection to a step change in Po_2 (0 to 100 % O_2), at 20.0° C, was 0.35 sec (on) and 0.38 sec (off); it was independent of the electrolyte composition.

4. *Independency of CO_2 and oxygen sensing properties*

The influence of oxygen on the CO_2 sensing properties was studied previously (see chapter 1). Oxygen in moderate concentrations was shown to have no effect upon the E *vs* pH curve up to a pH value of about 7.5 at 25° C. Moreover, calibration of the CO_2 probe with gas mixtures consisting of CO_2 and air instead of nitrogen gave similar E *vs* log Pco_2 lines as presented in figure 2.2.
The influence of CO_2 on the oxygen sensing properties was investigated by studying the effect of CO_2 in various concentrations on the polarogram, the calibration line and the response time for oxygen.
The oxygen polarogram appeared to be unaffected by CO_2. $E_{\frac{1}{2}}$ values remained unchanged within 40 mV for electrolyte solutions consisting of quinhydrone or quinhydrone with bicarbonate. Figure 2.4 demonstrates that in both cases

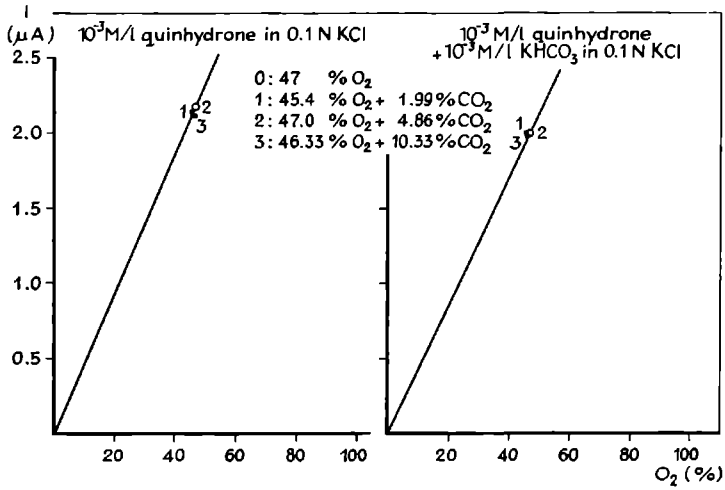


FIGURE 2.4 Oxygen calibration lines in the presence of various CO_2 concentrations. Electrolyte composition is 10^{-3} M/l quinhydrone in 0.1 N KCl and 10^{-3} M/l quinhydrone with 10^{-3} M/l $KHCO_3$ in 0.1 N KCl, resp.

CO_2 does not interfere with the oxygen calibration line (cathode surface area 0.07 mm^2).

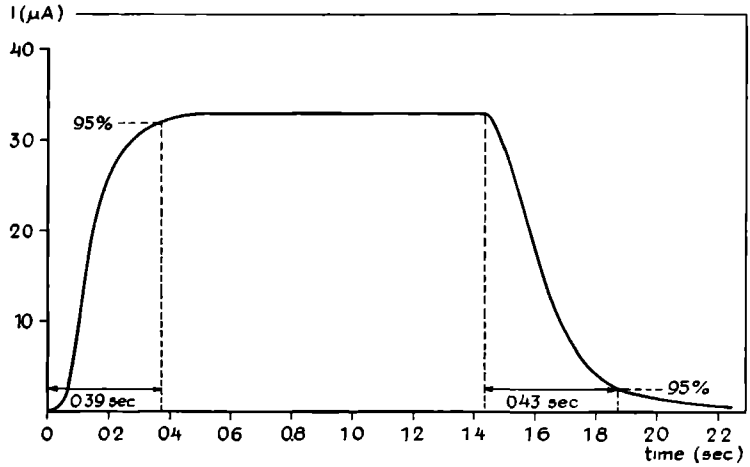


FIGURE 2.5 Response time of the CO_2 probe used as an oxygen electrode. The electrode covered by 6 μ teflon is exposed to a step change in both oxygen (0 % to 43.48 %) and CO_2 concentration (0 % to 25.58 %). Response time for 95 % deflection is 0.39 sec (on) and 0.43 sec (off) at 20.0° C.

As shown in figure 2.5 for the probe with a cathode area of 2.5 mm^2, the response time for 95 % deflection to a step change of Po_2 in the presence of CO_2 does not differ greatly from that in the absence of CO_2 (see preceding section; oxygen concentrations 0–43.48 %, CO_2 concentrations 0–20.58 %; 6 μ teflon, 10^{-3} M/l quinhydrone in 0.1 N KCl, 37.0° C).

DISCUSSION

No specific effect of the shape of the metal electrode was observed in agreement with data of Livingston, Morgan, Lammert *et al.* (1931). According to these authors, however, the size of the metal electrode definitely affects stability and reproducibility. Platinum foils of dimensions larger than 1 cm^2 are recommended, which gave potential differences of less than 10 μV when measured against one another. If the quinhydrone electrode is to produce an oxidation-reduction potential according to its electrode equation it will be clear that neither the activity of quinone and hydroquinone nor that of the metal phase can be

reduced indefinitely. At very small surface areas the exchange of charge across the metal-solution interface is expected to be impaired, leading to deviations from ideal electrode behavior. Apart from becoming polarizable the electrode will be unable to provide enough current at the proper potential to feed any recording instrument. For the same reasons the volume of solvent in contact with the metal phase cannot be restricted indefinitely. It appears from table 2.1 that this lower limit may be reached in the blank platinum electrode at a surface of about 1 mm^2. The discontinuity in the E *vs* pH curve observed between pH 5.5 and 6.5 might be a consequence of the two principal reaction mechanisms, each operating in a different pH range (below pH 5 and above pH 6) as proposed for the quinhydrone electrode process by Vetter (1952).

The instability of CO_2 response observed in the membrane-covered microelectrode is at least partially due to the relatively small area of the metal surface (stability is improved upon platinizing) but may as well arise from imperfections in design. In view of this drift, CO_2 sensing properties have not been studied thus far in the presence of bicarbonate. Response time for CO_2 in the microelectrode is somewhat faster than in the macroelectrode, due to the reduced layer thickness of the electrolyte solution and the correspondingly lower capacity to take up CO_2.

Concerning its oxygen sensing properties, the probe shows most of the features, including high stability and fast response, as met in the oxygen microelectrode (Beneken Kolmer and Kreuzer, 1968). The relatively large cathode area of the probe (35 times as large as in the oxygen microelectrodes) will increase its oxygen consumption and hence its dependency on flow of the medium, thus making it suited primarily for application in the gas phase. Any compromise in this respect may hardly be achieved without membranes combining high permeation rate for CO_2 (in order to get a sufficiently fast response) with poor permeability to oxygen.

In section 4 of Results it is shown that CO_2 and oxygen sensing properties are not affected by the presence of oxygen and CO_2 respectively. It remains to be evaluated, however, whether this independency is still maintained when CO_2 and oxygen tensions are to be measured in rapid succession. It seems improbable that oxygen determination will be impaired by a preceding CO_2 estimation but application of a negative voltage bias sufficiently high to reduce oxygen may at the same time reduce a small amount of quinone, thus interfering with CO_2 determination.

Polarography of quinhydrone solutions has been studied extensively by Müller

(1940). With the dropping mercury electrode the halfwave potential of quinone and hydroquinone in unbuffered solutions appeared to be dependent on the concentration of both redox components as well as on the pH of the solution. These effects were shown to result from pH changes at the electrode interface. Similar phenomena were observed when platinum electrodes were used. Besides, quinhydrone solutions occasionally appeared to behave irreversibly with respect to these electrodes, especially when surface areas were small (Laitinen and Kolthoff, 1941; Müller, 1947).

In oxygen polarograms taken in unbuffered quinhydrone solutions no evidence for quinone reduction was observed (Figure 2.3). Preliminary experiments using a commercial polarograph (Metrohm) and a rotating platinum electrode failed to show any cathodic reduction of quinone in freshly prepared unbuffered quinone and quinhydrone solutions (10^{-3} M/l in 0.1 N KCl). More evidence is needed, however, to evaluate the oxidation-reduction state of quinhydrone solutions during oxygen polarography.

Recently a solid reversible quinone electrode has been prepared by mixing quinone with gold or graphite (Alt *et al.*, 1971). Solid quinhydrone electrodes prepared in a similar way with platinum or gold may be advantageous in oxidation-reduction potentiometry and in oxygen polarography as well.

SUMMARY

A membrane-covered CO_2 microelectrode system is presented which consists of a platinum electrode surrounded by a ring-shaped Ag-AgCl electrode, both in contact with an electrolyte layer composed of 10^{-3} M/l quinhydrone in 0.1 N KCl. The resulting oxidation-reduction potential is shown to vary linearly with the logarithm of the Pco_2 of the medium. Response time for 95 % deflection to a step change in Pco_2 of 65 mm Hg is about 1 min, i.e. considerably less than in the macroelectrode described previously, but stability is decreased at the same time. Since oxygen in concentrations up to about 20 % (air) did not influence the CO_2 response and the presence of both CO_2 (10.33 %) and quinhydrone (10^{-3} M/l) did not alter the oxygen polarogram, this electrode may be used independently as an oxygen electrode as well. Stability and response time for oxygen were similar to those of the Clark electrode. Possibilities and limitations for in vivo estimation of Pco_2 and Po_2 are discussed.

Chapter 3

THE CO_2 CONDUCTIVITY ELECTRODE, A FAST RESPONDING CO_2 MICROELECTRODE

INTRODUCTION

In the previous chapters a new method was presented for the estimation of CO_2 partial pressure (Pco_2) in gases and liquids. The response time of an electrode based on this method as well as of the conventional Stow electrode (Stow, Baer and Randall, 1957) is determined by the time necessary to establish CO_2 equilibrium in the electrolyte solution. It mainly depends on the CO_2 permeation rate of the membrane and the capacity of the electrolyte solution for CO_2. With a teflon membrane of 6 μ thickness (which is reduced to about 80 % by stretching it on the electrode) and an electrolyte layer of 50 μ thickness, a time constant $t = RC$ of about 10 seconds is obtained; R refers to the diffusion resistance of teflon (6.3 to 12 mm Hg/(mole. second^{-1}) for each mm^2 of its membrane surface) and C is obtained from the solubility of CO_2 in water ($3.3\ 10^{-5}$ M/l. mm Hg). In evaluating this time constant no allowance has been made for the rather slow rate of hydration of CO_2, which will prolong it considerably especially at low Pco_2 values ($Pco_2 < 10$ mm Hg; Lunn and Mapleson, 1963).

It is possible to construct electrodes with a smaller time constant either by reducing the thickness of membrane and electrolyte layer or by using membranes more permeable to CO_2. For instance an electrode with a 25 μ silicone rubber membrane (Silastic S 2000), reduced to 20 μ after being mounted on the electrode tip, and an electrolyte layer of 100 μ, should have a time constant of 0.5 seconds only. In preliminary experiments, using commercially available micro-glass electrodes (Philips) or glass electrodes of own design as pH indicator, such CO_2 electrodes appeared to be unstable and showed a very short lifetime.

In the present chapter a CO_2 microelectrode system is presented in which the principle of establishing a CO_2 equilibrium between electrolyte layer and medium is abandoned. The time saved in this way reduces the time constant of

the system to about 3 seconds, thus providing a response time of 10 seconds for 90 % deflection.

PRINCIPLE

The permeation rate of CO_2 through a membrane depends, apart from membrane properties and temperature, on the pressure difference across the membrane. If the Pco_2 at the electrode side of the membrane is kept constant and very low (i.e. $Pco_2 = 0$) the permeation rate will depend on the Pco_2 of the medium only. Inasmuch as loss of CO_2 from the medium due to membrane diffusion has no effect upon its Pco_2, determination of the CO_2 permeation rate through the membrane will be indicative for the Pco_2 of the medium.

In practice this may be achieved by exposing a membrane-covered electrode, which is continuously flushed with a CO_2-free carrier gas or liquid at a constant rate, to a medium of unknown Pco_2. After having passed the membrane CO_2 is taken up by the carrier and transferred to a fast responding CO_2 analyzer for continuous estimation of the CO_2 concentration in the carrier gas or liquid. The permeation rate thus obtained may be related to the Pco_2 of the medium by calibrating the electrode system with gases of known Pco_2.

When using a gas phase as a carrier, CO_2 concentration may be determined by mass spectrometry, thermal conductivity or infrared analysis. A major disadvantage however is that membranes permeable to CO_2 transmit most other gases as well. The medium will thus be contaminated by the carrier gas which is particularly troublesome when Pco_2 is estimated in vivo. Both teflon and silicone rubber are about 3000 times less permeable to water vapor than to CO_2. The permeation rate of silicone rubber for water vapor is still considerable but this is less inconvenient since most biological media are saturated with water vapor. Therefore a liquid phase (water) has been chosen as a carrier.

Electrolyte conductivity has proven to be a fast, sensitive and accurate method for determination of CO_2 content in liquids. It has been demonstrated that in a 10 ml sample 0.13 µmole of CO_2 could be estimated with a standard deviation of 0.005 µmole (van Bruggen and Scott, 1961). Measurements of still higher accuracy have been reported by Maffly (1968). Conductometry has hardly been applied to the estimation of Pco_2 in biological systems. So far devices have been reported for determination of Pco_2 in gases and liquids (Murakami *et al.*, 1965) and in blood (Tsao and Vadnay, 1964). The microelectrode system presented here is a first attempt to apply conductometry to continuous monitoring of Pco_2 in vivo.

Construction of a CO_2 electrode prototype

Basic design

A stainless steel tip is attached to one end of an X ray double-lumen polyethylene catheter (no 2024p, American Cystoscope Makers Inc., New York) of 60 cm length and with an outer diameter of 2.7 mm and a lumen diameter of 0.8 mm. A CO_2 permeable membrane (Silastic S 2000 25 μ) is mounted on this tip by a device specially designed for this purpose and fixed by a stainless steel ring.

At the other end of the catheter each lumen is separately connected to a conductivity cell. The whole electrode system is flushed continuously with bidistilled water at a constant flow rate. Electrolyte conductivity of the water is measured before entering the catheter and after having passed the membrane. The difference in conductivity between inflowing and outflowing water is related to the Pco_2 of the medium to which the electrode is exposed.

Electrode tip

The tip used in the CO_2 electrode prototype is shown in figure 3.1. The sensitivity of the electrode system ($d\kappa/d\ Pco_2$), which is the change in specific conductivity (κ) of the carrier by a change in Pco_2 of the medium, is determined, among other factors, by the efficiency of CO_2 transfer from medium to carrier. It depends on membrane surface area, contact time of the carrier with the mem-

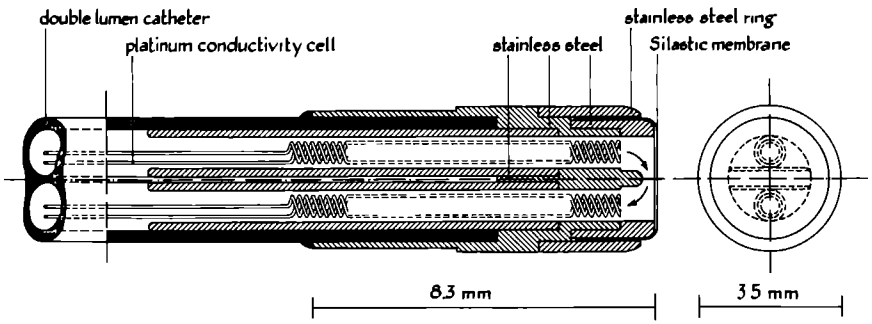


FIGURE 3.1 Stainless steel electrode tip covered by a CO_2 permeable membrane provided with a conductivity cell (cell f) and mounted on a flexible catheter.

brane, and carrier volume per unit membrane surface area (carrier layer thickness). In this electrode prototype a membrane surface area of 3.5 mm^2 and a layer thickness of 250 μ were chosen. Resistance to flow was approximately constant along the catheter and bulging of the membrane was avoided. Carrier flow rate was chosen within a range of 1 to 7 ml/min, thus giving contact times between 0.05 and 0.007 second. This limits at the same time the frequency of Pco_2 changes in the medium which the electrode system is able to follow.

Conductivity cell

The choice of the cell constant depends on the specific conductivity to be measured. Since at a specific conductivity of the order of $\mu ohm^{-1}\ cm^{-1}$ varia-

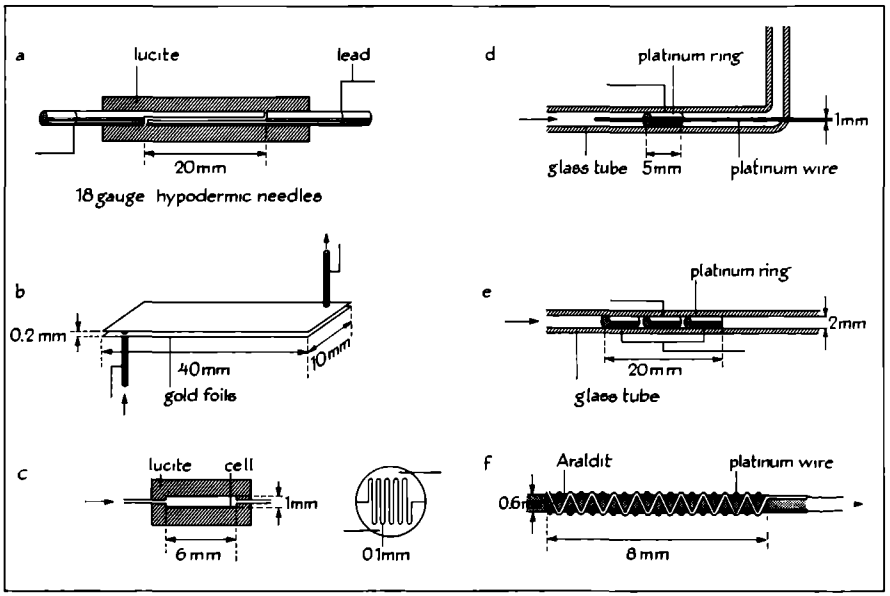


FIGURE 3.2 Series of conductivity cells used in developing the CO_2 electrode system.

a two 18 gauge stainless steel hypodermic needles (with luer-lok connection) filed off to half the diameter and inserted into a lucite block at a distance of 0.2 mm.

b two gold foils with a surface area of 400 mm^2 each, at a distance of 0.2 mm.

c two rhodinized brass foils of 14 mm^2 surface area each, at a distance of 0.1 mm.

d one platinum wire (diameter 1 mm) and ring (inner diameter 2 mm, length 5 mm) sealed into a glass tube.

e three platinum rings (inner diameter 2 mm, length 5 mm) sealed into a glass tube at a distance of 2 mm.

f two platinum wires (0.2 × 0.05 mm) coated by Araldit and wound at a distance of 0.2 mm to a coil of 0.6 mm inner diameter and a length of 8 mm.

tions of a few percent have to be determined, a conductivity cell with a low cell constant is required.

The cell volume should be close to that of the carrier in contact with the membrane in order to maintain the upper limit of the frequency at which Pco_2 fluctuations of the medium can be followed by the electrode. In figure 3.2 a series of cells are shown which have been used in developing the CO_2 electrode system. Most experiments reported here were carried out with cell d which is, due to its geometry, more suited than cells b and c to serve as a flow-through cell.

The construction of cell f deserves special attention since it has been designed in such a way as to fit the lumen of the catheter tip. The main advantage of this location is the reduction of response time and the convenience of temperature control. Platinum wires of 0.1 mm diameter were stretched to 0.05×0.2 mm and insulated by coating with Araldit. Two wires were wound at a distance of 0.2 mm to a coil of 0.6 mm inner diameter and a length of 8 mm. The coil was coated externally with Araldit. The interior of the coil was stripped of insulating material. Leads of 0.1 mm diameter were soldered to each wire. The cell thus obtained was inserted into the electrode tip with the leads passing through the catheter lumen.

Cell constants were determined either by using KCl standard solutions (Jones and Bradshaw, 1933) or by applying cells of known cell constant. In order to avoid adsorption of material and formation of large boundary layers at the cell surface, cells were kept free of platinum black deposits. This also protects them from short-circuiting. After use the cells were filled with Hibitane 0.2 % to prevent microbial contamination.

Alternating current conductometry

In most experiments electrolyte conductivity was determined with a commercially available direct reading conductivity meter (Radiometer CDM 2, frequency 70 Hz, test voltage 0.25 V) connected to a two channel recorder (Rikadenki). Conductivity meter and recorder were calibrated by precision resistance boxes. In this way it was possible to estimate variations in specific conductivity of up to 0.02 $\mu ohm^{-1}\ cm^{-1}$. Specific conductivity was measured with the same cell before and after exposing the electrode to CO_2.

A further improvement was achieved by applying a conductivity bridge which includes both measuring and reference cell. This makes it possible to detect

directly variations in impedance due to CO_2 and reduces difficulties of eliminating A-C interference (capacitance effects).

Temperature control

The majority of aqueous electrolyte solutions have a temperature coefficient, with respect to their conductivity, of about 2 percent per degree at 25° C. When absolute conductivity values are to be obtained accurate temperature control is required. Estimation of relative values, i.e. of variations in conductivity as occurring in the CO_2 electrode, is expected to be less influenced by temperature changes and only as far as processes affecting these variations are concerned (diffusion and chemical reaction of CO_2).

The electrode was protected from fluctuations in ambient temperature by covering the catheter with Tygon tubing. The temperature difference along the catheter was measured by thermocouples built into the electrode tip and the conductivity cells and was checked to be constant to within 0.2° C. The temperature of inflowing and outflowing water was measured to 0.1° C with an Electric Universal Thermometer (type TE 3, Ellab).

Regulation of flow rate

The double lumen catheter provided with a silicone rubber membrane (Silastic S 2000 25 μ) was flushed with bidistilled water at a constant hydrostatic pressure difference. Flow rate was measured with a Rotameter and kept constant to within 0.2 ml/min. A needle valve was used in adjusting the flow rate to its required value.

Gas mixtures

CO_2-nitrogen gas mixtures were prepared from pure gases by mixing appropriate amounts in a gas mixing pump (Wösthoff type M 300/a).

RESULTS

1. *Electrolyte conductivity of water in equilibrium with CO_2 at 25.0° C*

5 ml of bidistilled water were introduced into an all-glass double-walled tonometer thermostated at 25.0 ± 0.2° C and equilibrated with gas mixtures of various CO_2 concentrations (0–10 %). Equilibration was performed by shaking

the tonometer on a linear shaker and flushing it continuously with a gas phase containing CO_2. After equilibrium was established electrolyte conductivity of the water was determined with a conductivity cell mounted in the wall of the tonometer. The cell constant was 0.18 $\pm$ 0.01 cm^{-1}.

Figure 3.3 shows a plot (a) of the specific conductivity κ ($ohm^{-1}cm^{-1}$) of water in equilibrium with CO_2 against partial CO_2 pressure. The calculated curve (b) was obtained from equation (4) (see discussion) using values for the equivalent ionic conductivity at infinite dilution (λ, $ohm^{-1}cm^2$ equivalent^{-1}) as given in table 3.1. In the lower range of Pco_2 ($Pco_2 < 10$ mm Hg) experimental and calculated curves agree reasonably well whereas at higher Pco_2 values a discre-

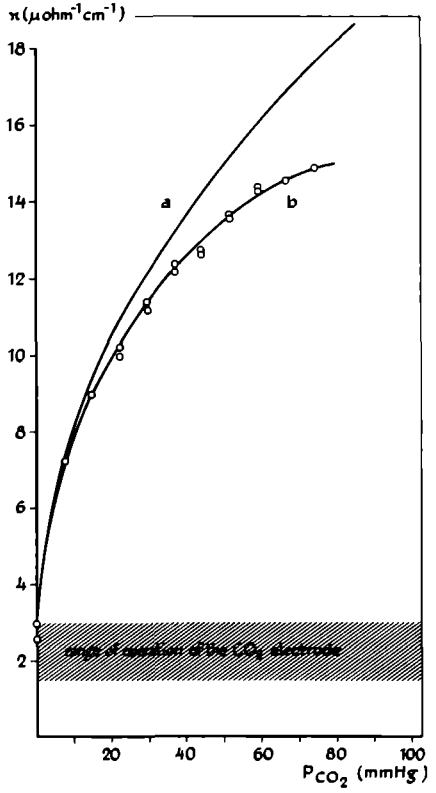


FIGURE 3.3 Specific conductivity ($\mu ohm^{-1}cm^{-1}$) of bidistilled water in equilibrium with CO_2 at varying partial CO_2 pressure. Temperature is 25.0°C.

a plot of experimental values.

b calculated curve obtained from equation (4).

TABLE 3.1 Relevant data related to CO_2 water equilibrium at 25.0°C.

	Value	Reference
K_1'	$10^{-6.352}$	Harned and Davis, 1943
K_2	$10^{-10.329}$	Harned and Scholes, 1941
k_{CO_2}	0.0375 sec^{-1}	(k_{31}') Gibbons and Edsall, 1963
k_{HCO^-}	5.5 10^4 M^{-1} sec^{-1}	(k_{13}') Gibbons and Edsall, 1963
$\lambda^o_{H^+}$	349.8 $ohm^{-1}cm^2$ $equiv.^{-1}$	Robinson and Stokes, 1959
$\lambda^o_{OH^-}$	198.3	Robinson and Stokes, 1959
$\lambda^o_{HCO_3^-}$	44.5	Robinson and Stokes, 1959
$\lambda^o_{CO_3^=}$	69.3	Robinson and Stokes, 1959

pancy is observed which grows at increasing P_{CO_2}. This discrepancy is largely due to the assumptions made in deriving equation (4).

2. *Properties of the CO_2 conductivity electrode*

a. Estimation of P_{CO_2} in the gas phase

Figure 3.4 presents the relation between specific conductivity κ and P_{CO_2} at

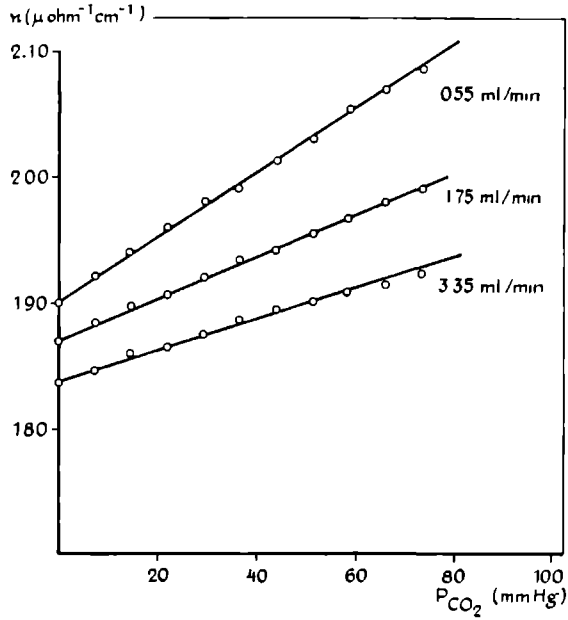


FIGURE 3.4 Specific conductivity κ of the carrier at three different flow rates (0.55, 1.75 and 3.35 ml/min) after exposure of the electrode to a gas phase of varying CO_2 partial pressure. Temperature is 18.0°C.

three different flow rates (0.55, 1.75, 3.35 ml/min) and at a temperature of 18.0°C (i.e. the temperature of the water leaving the catheter). This relationship appears to be linear within the Pco_2 range studied. The sensitivity of the electrode is defined by the slope $\Delta\kappa/\Delta Pco_2$ of these lines. An increase of flow rate lowers the specific conductivity at constant Pco_2 as well as the sensitivity. The reduction of κ at $Pco_2 = 0$ is due to the decreasing conductivity of the carrier at higher flow rates. It probably originates from boundary effects on the surface of the conductivity cell. The sensitivity for CO_2 is diminished at higher flow rates because both contact time of the carrier with CO_2 at the membrane and time available for hydration of CO_2 are shortened.

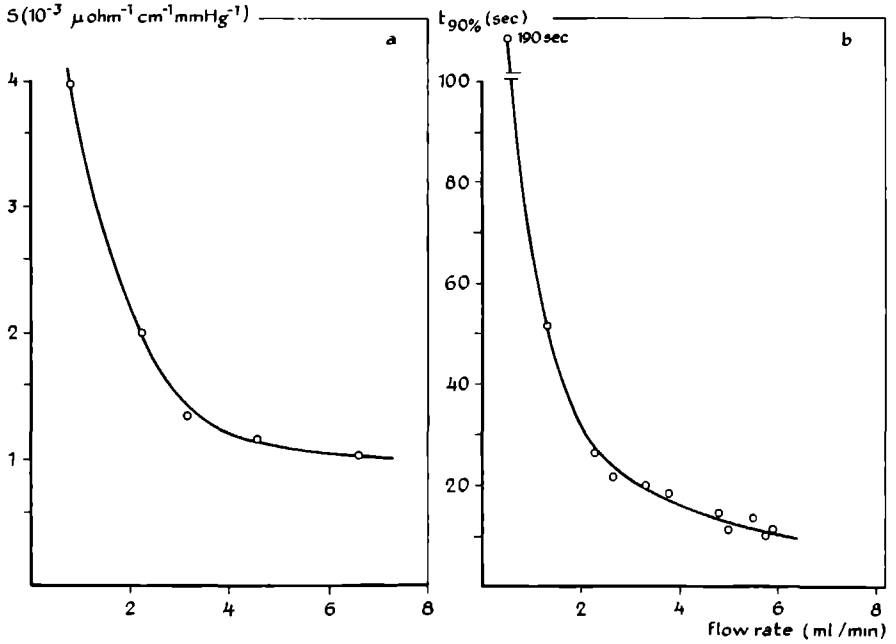


FIGURE 3.5a, b Sensitivity (a) ($\Delta\kappa/\Delta P_{CO_2}$) and response time (b) for 90 % deflection of the electrode system at various flow rates of the carrier. The electrode is exposed to a gas phase of 10 % CO_2 in nitrogen. Cell d is located at a distance of 60 cm from the membrane.

Sensitivity and response time for 90 % deflection are given in figure 3.5a and b at various flow rates of up to about 6 ml/min (cell d at 60 cm from the membrane). High sensitivity and fast response oppose each other. At high flow rates (> 5 ml/min) the response time for 90 % deflection is about 10 seconds whereas the sensitivity is rather low (1 $\mu ohm^{-1}cm^{-1}$ mm Hg^{-1}).

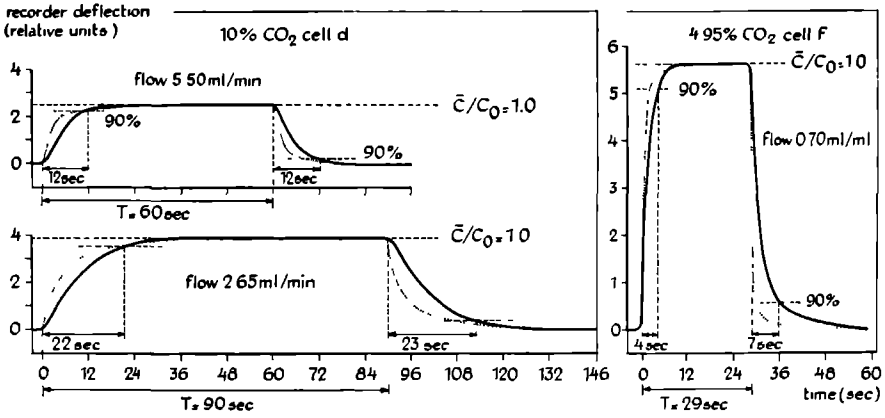


FIGURE 3.6 Time course of the CO_2 electrode

a provided with cell d at a distance of 45 cm from the membrane. Carrier flow rates are 5.50 and 2.65 ml/min.
The electrode is exposed to a step change of P_{CO_2} between 0 and 10 % CO_2.

b provided with cell f at a distance of within 10 mm from the membrane. Carrier flow rate is 0.70 ml/min.
The electrode is exposed to a step change of P_{CO_2} between 0 and 4.95 % CO_2.

(————) actual time course of the electrode

( ) time course calculated from the model presented in the discussion.

Figure 3.6a and b shows the time course of the CO_2 electrode upon a step change in P_{CO_2} (0–10 %) at two different flow rates (2.65 and 5.50 ml/min). The electrode is provided with cell d at a distance of 45 cm from the membrane.
In figure 3.6b the time course of the electrode is shown when provided with cell f at a distance of within 10 mm from the membrane. Carrier flow rate is 0.70 ml/min. Dotted lines indicate the calculated time course based on the model given in the discussion. Actual and calculated response times for 90 % deflection rise with decreasing flow rate and increasing distance from the membrane.

b. Estimation of P_{CO_2} in the liquid phase

Water was equilibrated in a tonometer thermostated at 18.0 ± 0.2°C with gas mixtures of various CO_2 concentrations (0–50 % CO_2). The P_{CO_2} electrode was mounted in the wall of the tonometer. By rotating the tonometer it was possible to measure alternately P_{CO_2} in gas and liquid phase.
The sensitivity of the electrode in the liquid phase was of the same order of magnitude as in the gas phase but appeared to be dependent on the frequency of displacement exerted by the shaker. For instance at a frequency of 45 trans-

lations per minute a linear relation between P_{CO_2} and specific conductivity was obtained with a slope of 2.10^{-3} $\mu ohm^{-1}cm^{-1}$ mm Hg^{-1}, i.e. about the same as in the gas phase (carrier flow 2.20 ml/min). At lower frequencies sensitivity decreased considerably (to 50 % at a frequency of 0). This reduction in sensitivity may largely result from the existence of CO_2-poor boundary layers near the membrane of the electrode. The extent of these boundary layers is influenced by the motion of the bulk fluid in the neighborhood of the electrode tip.

DISCUSSION

1. *The CO_2 water system in equilibrium*

The specific conductivity of an electrolyte solution at constant temperature is determined by the concentration and the mobility of the electrolytes present according to

$$\kappa = \frac{1}{1000} \sum_{i=1}^{n} C_i \lambda_i \qquad (1)$$

where C_i is the concentration of the i^{th} ion in equivalents per liter, λ_i its equivalent conductivity in $ohm^{-1}cm^2$ per equivalent and n the number of ionic species present in solution. If equivalent conductivity is taken to be independent of concentration which is permitted only at infinite dilution equation (1) becomes

$$\kappa = \frac{1}{1000} \sum_{i=1}^{n} C_i \lambda^\circ_i \qquad (2)$$

where λ°_i is the equivalent conductivity of the i^{th} ion at infinite dilution. The specific conductivity of water in equilibrium with CO_2 is then given by

$$10^3 \kappa = C_{H^+} \lambda^\circ_{H^+} + C_{OH^-} \lambda^\circ_{OH^-} + C_{HCO_3^-} \lambda^\circ_{HCO_3^-} + C_{CO_3^{=}} \lambda^\circ_{CO_3^{=}} \qquad (3)$$

Since under circumstances as met in figure 3.3 $\lambda^\circ_{HCO_3^-} \gg \frac{2K_2}{C_{H^+}} \lambda^\circ_{CO_3^{=}}$

(K_2 refers to the second dissociation constant of carbonic acid), the contribution of carbonate ions to the specific conductivity of the solution may be neglec-

ted. When applying the equilibrium conditions for water and carbonic acid equation (3) becomes

$$10^3\kappa = (K'_1\,[CO_2] + K_w)^{\frac{1}{2}}\lambda^\circ_{H^+} + K_w\,(K'_1\,[CO_2] + K_w)^{-\frac{1}{2}}\lambda^\circ_{OH^-} + K'_1\,[CO_2]\,(K'_1\,[CO_2] + K_w)^{-\frac{1}{2}}\lambda^\circ_{HCO_3^-} \quad (4)$$

K'_1 is the apparent first dissociation constant of carbonic acid and K_w the ionization constant of water. Substitution of appropriate values from table 3.1 gives the calculated curve of figure 3.3.
At low P_{CO_2} values, i.e. in the range where the CO_2 electrode is operating, agreement is good, at high P_{CO_2} values ($P_{CO_2} > 10$ mm Hg) a discrepancy is observed between experimental and calculated values. This may largely be due to assuming the equivalent conductivity to be independent from concentration.

2. *The CO_2 electrode, some theoretical considerations*

In the CO_2 electrode as described here a number of processes are involved which proceed simultaneously or in rapid succession. Diffusion is involved in the transition of CO_2 from medium to carrier (membrane diffusion) and during transport to the conductivity cell. CO_2 and its ions are transferred to the conductivity cell by convection, a hydrodynamic process which is characterized by nature of flow, velocity profile, local turbulence, boundary layers, etc. Dissolved CO_2 will take part in a chemical reaction in which it combines with the carrier and dissociates partially into ions. This hydration reaction proceeds relatively slowly compared with most ionic reactions. Each of these processes will have its own impact on design and properties of the electrode system and will therefore be considered in some detail.

a. Membrane diffusion

The volume of CO_2 which traverses the membrane in a certain time is readily obtained from the P_{CO_2} difference across the membrane and the dimensions and CO_2 permeability characteristics of the membrane material used (Silastic S 2000: $0.33.10^{-6}$ ml CO_2.cm.sec^{-1}.cm^{-2}.cm Hg^{-1}). If the electrode is exposed to a gas phase with a P_{CO_2} of 0.5 mm Hg for 0.01 sec, about 23.10^{-10} ml CO_2 will permeate the membrane. At a carrier flow rate of 5 ml/min this will give a CO_2 concentration in the water of about 10^{-7} M/l. Conditions as given here

refer to requirements which are to be satisfied by an electrode system almost optimally suited for in vivo P_{CO_2} monitoring.

So far the existence of a hydrodynamic boundary layer arising from adherence of a streaming fluid to the membrane surface has been neglected. This boundary layer of finite thickness dependent on the flow rate of the fluid may add to the diffusion resistance of the membrane. Since diffusion resistances of silicone rubber and water are of the same order of magnitude, a boundary layer will exist at the membrane-carrier interface which will lower the CO_2 concentration in the bulk of the carrier. A further reduction of CO_2 transfer from medium to carrier will arise when P_{CO_2} is measured in a liquid medium (water, plasma). As was observed in section 2b of Results the thickness of the boundary layer at the medium-membrane interface and thus the permeation rate of CO_2 from medium to carrier depend on the velocity of fluid along the membrane. When P_{CO_2} is estimated in the gas phase diffusion resistance of the medium may be neglected with respect to that of the membrane.

b. Convection

The mean velocity ($\bar{v}$) of the carrier water, i.e. the volume flow divided by the cross sectional surface area, is 200 mm/sec or less. With a tube diameter of 0.8 mm Reynolds' number will always be lower than 200. The flow pattern in the catheter is therefore expected to be laminar except for local disturbances at the electrode tip and the conductivity cell.

A model for the convection of CO_2 by laminar flow is shown in figure 3.7a, b. In a circular pipe of radius R the velocity v at a distance r from the axis is given by

$$v_r = v_0 (1 - r^2/R^2) \tag{5}$$

where v_0 is the maximum velocity (Figure 3.7a). If at $t = 0$ a certain amount of CO_2 is introduced instantaneously and distributed homogeneously over the cross-section at $x = 0$, it will after t_1 seconds be dispersed according to

$$x = v_0 t_1 - \left(1 - \frac{r^2}{R^2}\right) \tag{6}$$

If addition of CO_2 is continued at a constant rate for T seconds CO_2 concentration within this paraboloid will be constant and equal to $[CO_2]_0$, the CO_2

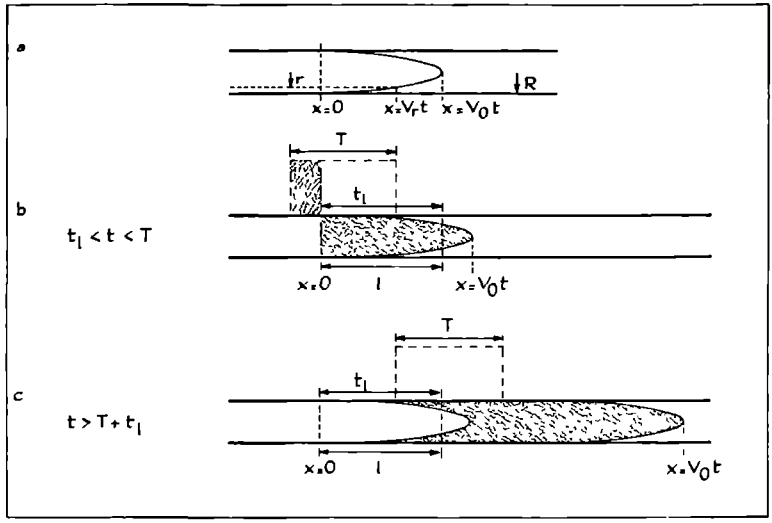


FIGURE 3.7.a, b, c Model for the dispersion of CO_2 by laminar flow in a circular pipe (radius R). Details are given in the next.

concentration in cross-section x = o at t = o. The mean CO_2 concentration in cross-section x where $o < x < v_o t_l$ is given by

$$(r_x/R)^2\,[CO_2]_o \quad \text{so} \tag{7}$$

$$[\overline{CO_2}]_x = [CO_2]_o \left(1 - \frac{x}{v_o t_l}\right)$$

If as shown in figure 3.7b, $[\overline{CO_2}]_x$ is considered in a cross-section at a fixed distance x = l for a time interval $l/v_o < t < T$ then

$$[\overline{CO_2}]_l = [CO_2]_o \left(1 - \frac{t_l}{t}\right) \tag{8}$$

where $t_l = l/v_o$.

Let l be the distance between membrane and conductivity cell (length of catheter) and t_l the minimum time delay at which CO_2 is carried from membrane to conductivity cell; the response time for 90 % deflection ($t_{90\%}$) may then be defined

as the time after t_l required to give $[CO_2]_l = 0.90\ [CO_2]_o$. From (8) it follows that $t_{90\%} = 9\ t_l$.

At $t > T + t_l$ (figure 3.7c) mean concentration of CO_2 at l is given by

$$[CO_2]_l = [CO_2]_o \frac{t_l T}{t(t - T)} \tag{9}$$

The time course of the electrode system upon a step change in P_{CO_2} may be derived from the model equations (8) and (9) by plotting $[\overline{CO_2}]/[CO_2]_o$ against t, where $t \gg l/v_o$ for on responses and $t \gg T + l/v_o$ for off responses. From figure 3.6a, b it appears that the change of $[\overline{CO_2}]/[CO_2]_o$ with time proceeds faster in the model curve than in the electrode system.

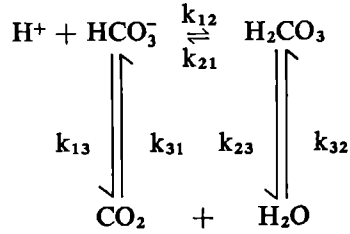
This difference may result from the single or combined effect of the following phenomena. The main cause of the discrepancy probably arises from the disturbing influence of the geometry of the conductivity cell on the flow pattern along the catheter. Since dimensions and shape of cell d are different from those of the catheter lumen the CO_2 profile will be blurred. This effect will almost be absent when cell f is used.

From conditions given by Taylor (1953) it follows that in the electrode system CO_2 is dispersed, apart from convection, by molecular diffusion as well. Longitudinal diffusion may be shown to distort the CO_2 paraboloid only negligibly, radial diffusion, however, which proceeds proportionally to $(D_{eff.}\ t)^{\frac{1}{2}}$ (where $D_{eff} = R^2 v_o^2 / 192\ D_{CO_2}$ and D_{CO_2} is the diffusion constant of CO_2 in water), will alter the CO_2 profile and thus the response time significantly.

The model deviates from the actual electrode process in another respect. During convection along the catheter CO_2 is reacting chemically with the carrier and the ions produced in this reaction are detected rather than CO_2. Since, as will be shown, the time constant of this reaction is of the same order of magnitude as t_l the concentration of the reaction products within the paraboloid will be time dependent rather than constant as assumed in the model. Hence hydration of CO_2 will affect the time course of the CO_2 electrode too.

c. Kinetics of CO_2 hydration

As suggested by Eigen, Kustin and Maass (1961), hydration of CO_2 and ionization of carbonic acid satisfy the following reaction scheme



This reaction mechanism turns into the classical scheme for $k_{13} = k_{31} = 0$. Since all values of velocity constants so far obtained are in terms of k_{23} and k_{32} only and full evidence for the Eigen scheme is not available at present, CO_2-water equilibrium will be treated classically according to:

$$CO_2 + H_2O \underset{k_1}{\overset{k_2}{\rightleftharpoons}} H_2CO_3 \tag{11}$$

$$H_2CO_3 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} H^+ + HCO_3^- \tag{12}$$

Combining rate equations (11) and (12) for H_2CO_3 and substituting $[H_2CO_3]$ thus obtained into the rate equation for HCO_3^- gives

$$\frac{d\,[HCO_3^-]}{dt} = \frac{k_1\,k_3}{k_2 - k_3}\,[CO_2]\,[H_2O] - \left(k_4 + \left(\frac{k_3\,k_4}{k_2 - k_3}\right)\right)[H^+]\,[HCO_3^-] \tag{13}$$

Equation (13) demonstrates that reactions (11) and (12) may be combined to

$$CO_2 + H_2O \underset{k_{HCO_3^-}}{\overset{k_{CO_2}}{\rightleftharpoons}} H^+ + HCO_3^- \tag{14}$$

where $k_{CO_2} = \dfrac{k_1\,k_3}{k_2 - k_3}\,[H_2O]$ and $k_{HCO_3^-} = k_4 + \dfrac{k_3\,k_4}{k_2 - k_3}$

The relevant equations to be considered in the CO_2 electrode process are

$$\frac{d\,[HCO_3^-]}{dt} = k_{CO_2}\,[CO_2] - k_{HCO_3^-}\,[H^+]\,[HCO_3^-] \tag{15}$$

and $$H^+ + OH^- \rightleftharpoons H_2O \qquad K_w \qquad (16)$$

Let at $t = 0$, $[CO_2]_o = a$, $[HCO_3^-]_o = 0$ and $[H^+]_o = [OH^-]_o = \sqrt{K_w}$ and at $t > 0$, $[HCO_3^-] = x$.

Suppose reaction (16) is always at equilibrium with respect to (15). If at a certain time t after contact with CO_2 y hydrogen ions have combined with hydroxyl ions then

$$[H^+]_t = [H^+]_o + x - y \text{ and } [OH^-]_t = [OH^-]_o - y$$

Applying (16) gives

$$[H^+]_t = \tfrac{1}{2}x + \tfrac{1}{2}\sqrt{x^2 + 4K_w} \qquad (17)$$

Substitution of (17) into (15) gives

$$\frac{dx}{dt} = k_{CO_2}(a - x) - \tfrac{1}{2}k_{HCO_3^-}x^2 - \tfrac{1}{2}k_{HCO_3^-}x\sqrt{x^2 + 4K_w} \qquad (18)$$

If $\sqrt{x^2 + 4K_w}$ is approximated by $x + 2K_w/x$, equation (18) may be solved for

$$x = \frac{A(1 - e^{-\gamma t})}{1 + C e^{-\gamma t}} \qquad (19)$$

where $\gamma = (k^2_{CO_2} + 4k_{CO_2}k_{HCO_3^-}a - 4k_{HCO_3^-}K_w)^{\frac{1}{2}}$

$$A = \frac{\gamma - k_{CO_2}}{2k_{HCO_3^-}}$$

$$C = \frac{\gamma - k_{CO_2}}{\gamma + k_{CO_2}}$$

Taking $a = 10^{-7}$ M/l CO_2 and for k_{CO_2} and $k_{HCO_3^-}$ the appropriate values at 25°C from table 3.1, $[HCO_3^-]$ at equilibrium (A) becomes $0.65\ 10^{-7}$ M/l. The time required to attain a fraction f of the equilibrium concentration is given by

$$t_f = \frac{1}{\gamma}\ln\frac{(1 + f) + k_5(1 - f)}{(\gamma + k_5)(1 - f)} \qquad (20)$$

$t_{\frac{1}{2}}$ is about 17 seconds and $t_{9/10}$ is 52 seconds in this situation.

It will be clear that both flow rate and distance l between membrane and cell determine the sensitivity of the electrode system. At constant flow rate sensitivity increases with l until equilibrium is reached. At constant l and varying flow rate sensitivity is determined by the initial CO_2 concentration, $[CO_2]_o$, in the carrier which is inversely proportional to the flow rate, and by t_f of the hydration reaction which depends on $[CO_2]_o$ and hence on flow rate as well. This will be illustrated in relation to data obtained under conditions similar to those of figure 3.5a, b.

Initial CO_2 concentration, $[CO_2]_o$, equilibrium HCO_3^- concentration, $[HCO_3^-]_{eq}$, and actual HCO_3^- concentration at l, $[HCO_3^-]_l$, were calculated at each flow rate using the rate constants of table 3.1.

In figure 3.8 the ratios $[HCO_3^-]_l/[HCO_3^-]_{eq}$ and $[HCO_3^-]_l/([CO_2]_o - [HCO_3^-]_l)$ as well as $t_{\frac{1}{2}}$ are plotted against the flow rate. At a given distance l there appears

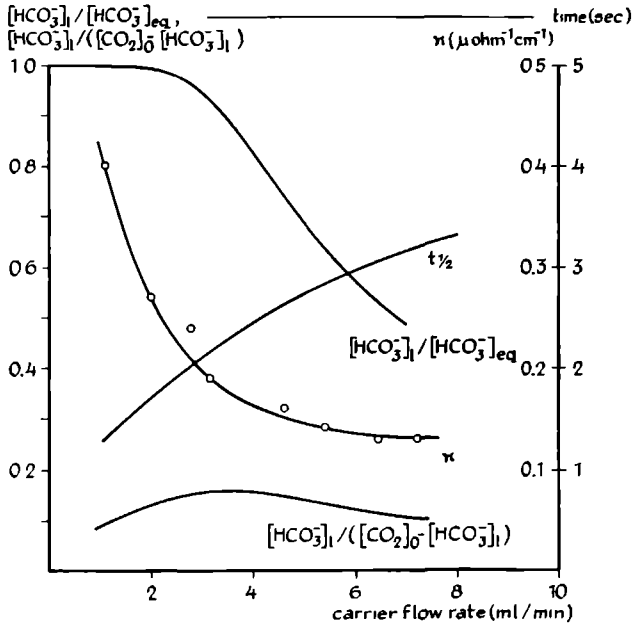


FIGURE 3.8 Relation between the specific conductivity κ and the carrier flow rate when the conductivity cell is located at a fixed distance l from the membrane (l = 60 cm). Values for the ratios $[HCO_3^-]_l/[HCO_3^-]_{eq}$ and $[HCO_3^-]_l/([CO_2]_o - [HCO_3^-]_l)$ and for the half-time constant of the CO_2 hydration ($t_{\frac{1}{2}}$) calculated from equations (19) and (20) are included.

to be a flow rate where the turnover of CO_2 into HCO_3^- is maximal. At lower flow rates CO_2 equilibrium is already established in the carrier when l is reached and any increase of $[CO_2]_o$ will result in a relatively small increase of $[HCO_3^-]_l$, thus reducing $[HCO_3^-]_l/[CO_2]_o$. The rise of t_f at higher flow rates will accordingly lower this ratio too. If $[CO_2]_o$ is of the order of 10^{-10} M/l equation (19) turns into $x = a\,(1 - e^{-k_{CO_2}t})$ and t_f becomes independent of a.
In the preceding section it was shown that, when a fast response is required, the conductivity cell should be located close to the membrane. If at the same time high sensitivity and low CO_2 consumption are needed (in order to reduce dependency on the flow of the medium), figure 3.8 shows that carrier flow rate may optimally be chosen in the range where turnover of CO_2 into bicarbonate is high.

3. *The CO_2 electrode, possibilities, limitations and prospects*

The main requirements to be satisfied by a sensor suited for continuous monitoring of P_{CO_2} *in vivo* in the field of respiratory physiology are, apart from dimensional aspects, high accuracy and fast response. The high accuracy (error 0.5 mm Hg or less) is required in view of the relatively small difference in P_{CO_2} between arterial and venous blood compared with that of oxygen and the fast response is needed in order to follow P_{CO_2} fluctuations at frequencies of about 10 sec^{-1} resulting from respiratory and circulatory effects.
The prototype presented here, although being far remote from this goal, seems to offer better possibilities in comparison with conventional techniques. With cells located at a fixed distance of 60 cm the fastest response obtained allowed to follow P_{CO_2} fluctuations of less than 0.05 sec^{-1}. When cells were placed within the electrode tip faster responses were achieved corresponding to frequencies between 0.1 and 0.2 sec^{-1}. Concerning the error involved, the electrode, although not meeting the requirements mentioned above, may show an error of well below 2 mm Hg P_{CO_2}, at least at low flow rates. A rather serious limitation of the electrode is its CO_2 consumption when P_{CO_2} is estimated in the liquid phase, which makes it highly dependent on the flow of the medium. This may be solved by using membranes with smaller surface area and/or lower permeability to CO_2. The reduction of sensitivity should be improved by other means.
Replacement of bidistilled by highly purified water (conductivity water) will increase carrier resistance and thus $\Delta\kappa/\kappa$. At lower conductivities, however,

balancing of the bridge becomes more difficult; this may partially be avoided by using cells of smaller cell constant. Problems encountered in alternating current conductometry, particularly concerning the design of conductivity cells and the elimination of capacitance effects, were reviewed extensively elsewhere (Robinson and Stokes, chapter 5 and references, 1959; Jones and Christian, 1935) and will not be discussed here. Further miniaturization of the electrode system is expected to increase sensitivity and reduce CO_2 consumption at the same time.

SUMMARY

A fast responding CO_2 microelectrode system is presented which consists of a double-lumen polyethylene catheter provided with a stainless steel catheter tip which is covered by a CO_2 permeable membrane, and connected with each lumen separately to a conductivity cell. The catheter is flushed with bidistilled water at a constant flow rate and conductivity of inflowing and outflowing water is measured. The change of conductivity in the water leaving the catheter after exposure of the electrode to a medium containing CO_2 is related to the P_{CO_2} of the medium. High sensitivity and fast response, both depending on flow rate of the carrier water, are shown to oppose each other. When cells are placed at a distance of 45 cm from the membrane response time for 90 % deflection is about 10 sec (at flow rates of 5 ml/min), but when located inside the electrode tip at a distance of within 10 mm from the membrane response time is reduced to about 4 sec. Hydrodynamical aspects concerning dispersion of CO_2 in the carrier water and reaction kinetics of CO_2 hydration are discussed.

Chapter 4

THE CARBAMATE EQUILIBRIUM OF BOVINE HEMOGLOBIN AT 37°C

INTRODUCTION

Hemoglobin-bound CO_2 (carbamate hemoglobin, carbamino-hemoglobin) contributes significantly to the carbon dioxide transport by mammalian blood (Roughton, 1964).

Recently Rossi-Bernardi, Pace, Roughton and van Kempen (1969) introduced a new method to quantitatively estimate the formation of carbamate compounds in hemoglobin solutions equilibrated with CO_2. By this method it seemed possible to eliminate most of the corrections inherent to Faurholt's barium precipitation technique as applied to hemoglobin solutions by Ferguson and Roughton (1934) and Ferguson (1936).

In the present chapter the possibilities and limitations of this new procedure were studied particularly also with respect to its denaturating effect on hemoglobin after contact with alkali, and the method was then applied to the carbamate equilibrium of bovine hemoglobin at 37°C.

METHOD AND MATERIALS

Principle

Carbamino-hemoglobin is not easily distinguished from hemoglobin without any bound CO_2 by conventional techniques. A necessary step is to separate the carbamate compound from other forms of CO_2 present in solution (dissolved CO_2, bicarbonate). The amount of carbamate may then be estimated by combination of standard gas analysis of CO_2 (van Slyke) with determination of hemoglobin concentration (hemiglobin cyanide).

Under physiological conditions of pH and P_{CO_2} hemoglobin-bound CO_2 is in rapid and highly reversible equilibrium with other constituents in solution. Any alteration of these equilibrium conditions as expected to occur during the separation procedure will inevitably change the original carbamate concentration.

Before starting separation, carbamate equilibrium is therefore stabilized by mixing the hemoglobin solution with strong alkali in a Hartridge-Roughton mixing chamber. Separation is then achieved by gel permeation and ion exchange chromatography.

Preparation of hemoglobin solution

Carbamate equilibrium was studied on bovine hemoglobin because of its high alkaline resistance compared with hemoglobin of other species (Haurowitz, Hardin, and Dicks, 1954). This makes it more suited for the experimental conditions met during carbamate determination.

Hemoglobin solutions were prepared by the method of Adair and Adair (1934) at 4°C from freshly drawn heparinized bovine blood. Erythrocytes were centrifuged (4000 r.p.m.), washed three times with isotonic saline, and hemolyzed by slow addition of ether (10 % v/v) and solid NaCl (10 % w/v). After centrifugation (3000 r.p.m. for 20 min) the upper layer containing the cell stromata was removed. The remaining part of the solution was dialyzed during 48 hours against a 0.1 M KCl solution which was changed every 12 hours. Any precipitated material which might appear during dialyzation was removed by centrifugation (15000 r.p.m. during 30 min). The hemoglobin solution so obtained (concentration 10–12 mM (Fe)/l) was deoxygenated with nitrogen, stored at 4°C in all-glass double-walled reservoirs under a positive nitrogen pressure of 50 cm Hg, and used within three weeks after preparation. The methemoglobin concentration determined according to Kaplan (1965) never exceeded 2–3 % of the total hemoglobin concentration.

Preparation of 'double-blocked' bovine hemoglobin

Bovine hemoglobin specifically modified at the terminal amino groups of both α and β chains ('double-blocked' derivative) was prepared by carbamylating these amino groups with cyanate under conditions slightly different from those used for horse hemoglobin (Kilmartin and Rossi-Bernardi, 1969, 1970). Details will be given in chapter 5.

Equilibration of hemoglobin solution

All experiments were carried out at constant hemoglobin concentration and constant ionic strength (0.18). Therefore 24 ml of the hemoglobin stock solution

were diluted to 30 ml by adding appropriate volumes of KOH, HCl and KCl solutions, all of 0.1 N strength, in varying proportions according to the pH value required. Equilibration was performed at constant temperature in a specially designed rotating tonometer thermostated at 37.0 ± 0.2°C and flushed continuously with a gas phase at the same temperature. In this way equilibration was reached within 10 minutes.

CO_2-nitrogen and CO_2-oxygen gas mixtures were prepared from pure gases by mixing appropriate amounts in a gas mixing pump (Wösthoff type M 300/a). The gas mixtures were warmed up to and saturated with water vapor at 37.0°C. After equilibration the hemoglobin solution was divided into two parts. One portion was used for estimation of pH (Radiometer pH microelectrode unit type E 5021 connected to a pH meter 22 with scale expander PHA 630), P_{CO_2} and P_{O_2} (Radiometer electrodes types E 5036 and E 5046 both connected to a PHM 27 pH meter), CO_2 and oxygen content (van Slyke apparatus), and hemoglobin concentration (Beckmann DU 2 spectrophotometer). The remaining portion was used for carbamate determination.

Preparation of other solutions

All reagents used were of analytical grade. For the preparation of the mixing and diluting solutions KOH and KCl were used exclusively because of the only minor alkaline error of the potassium ion on the glass electrode. For pH measurement in strong alkaline solution a combined glass electrode was used with a very small alkaline error (Radiometer GK 2351 B).

In preliminary experiments hemoglobin solution was mixed with alkali in a volume ratio of 10 to 1. The 1.2 N KOH solution necessary to reach a pH of 12 was freed from carbonate according to Davies and Nancollas (1950) or by the method of Albert and Serjeant (1962). In the final experiments, where a mixing ratio of 1 to 1 was applied, all carbonate in the mixing solution was removed completely by the carbamate procedure itself. Thus there was no need to free the mixing solutions of carbonate before use.

Resins were prepared according to the instructions of the manufacturers. 2,3-diphosphoglycerate was obtained from Boehringer Company.

Carbamate determination

The procedure was similar to that described by Rossi-Bernardi, Pace, Roughton and van Kempen (1969) and is presented schematically in figure 4.1.

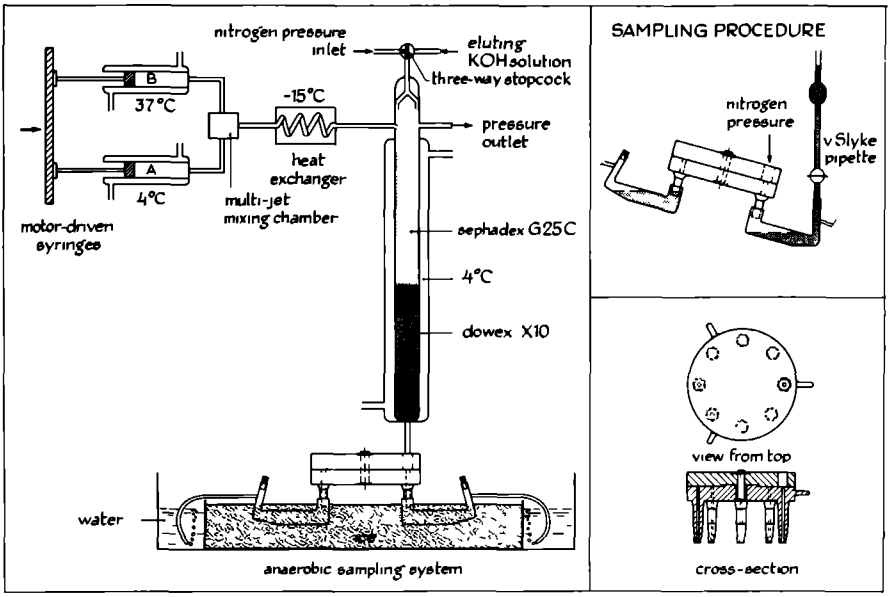


FIGURE 4.1 Experimental set-up for carbamate determination.

The contents of two syringes, one (B) loaded with about 5 ml of the equilibrated hemoglobin solution at 37.0°C and the other (A) with 0.5 N KOH at 4°C, were rapidly mixed by an electromotor in a Hartridge-Roughton 8-jet mixing chamber. The solution (pH = 12) was immediately cooled (4°C) after mixing and then anaerobically applied to a Sephadex-Dowex column thermostated at 4°C. Column dimensions are specified in Results.

A time relais was added by which the electromotor was activated during a fixed time interval. In this way the column was always charged with a constant volume of hemoglobin solution (4.5 ± 0.2 ml). Samples were collected anaerobically by an all-glass micro-fraction collector provided with 7 reservoirs which alternately could be connected to the column outlet by air-tight ground-glass joints.

From these reservoirs samples were transferred to the van Slyke apparatus (sampling procedure shown in figure 4.1) for estimation of total CO_2 and oxygen content. Hemoglobin concentration of each sample was determined as hemiglobin cyanide in a Beckmann DU 2 spectrophotometer calibrated according to van Kampen en Zijlstra (1961) with the international hemiglobin cyanide reference solution obtained from the Dutch Institute of Public Health.

All measurements were performed at least in duplicate but mostly in quadrupli-

cate. The standard deviation of the van Slyke determinations was always less than 0.01 for CO_2 contents ranging from 10 to 30 mM/l and 0.05 in the range of 0.5 to 2.0 mM/l (4 determinations). Similar values were obtained for oxygen. The standard deviation in the optical density (O.D.) readings of the hemiglobin cyanide solutions was 0.003 or less.
Hemoglobin spectra were scanned by a double-beam recording spectrophotometer (Hitachi Perkin Elmer model 124).
Computer calculations were carried out in a Bull General Electric computer with time sharing. Programs were written in BASIC language.

RESULTS

1. a. *The millimolar extinction coefficient of bovine hemiglobin cyanide at* 540 mμ

In contrast to human and horse hemoglobin (Zijlstra *et al.*, 1969) the millimolar extinction coefficient (ε) of bovine hemiglobin cyanide at 540 mμ is unknown. Since it is intended to study the stoichiometric relationship between hemoglobin and CO_2, an accurate estimation of hemoglobin concentration is required and and therefore $\varepsilon_{540\ m\mu}$ has to be evaluated.
Bovine hemoglobin solution was dialyzed against bidistilled water and deionized by passing it through a column with a mixed-bed ion exchange resin (Amberlite IRA 400 and IR 120). Part of this hemoglobin solution was converted to hemiglobin and O.D. was determined at 540 mμ. Dry weight was measured in one ml portions of the remaining part.
Taking a molecular weight of 16,085 per heme (Dayhoff 1970) for bovine hemoglobin, $\varepsilon_{540\ m\mu}$ as calculated from 26 estimations of dry weight and O.D. was 11.04 with a standard error of the mean of 0.04. This value is not significantly different from the generally accepted value for human hemoglobin (11.00, I.C.S.H. 1967) which therefore will be used also for bovine hemoglobin.

1. b. *The millimolar extinction coefficient of bovine hemiglobin cyanide at alkaline pH*

A key issue to be solved is whether the spectral properties of hemoglobin, especially its $\varepsilon_{540\ m\mu}$, are changed by the alkaline conditions met during carbamate estimation. Any alteration of $\varepsilon_{540\ m\mu}$ might affect subsequent hemoglobin concentration determination and thus the carbamate yield. Increasing amounts

of 4 N KOH were added to a hemiglobin cyanide solution of bovine hemoglobin by a microburette (Coleman). As soon as stable values were reached (which may take an hour or so at high pH values) O.D. was read at 540 mμ and pH was measured at the same time. Added volumes of KOH were so small that dilution could be neglected. The course of the O.D. at 540 mμ with increasing pH is shown in figure 4.2 (right side). Upon back titration with acid (4 N HCl) this change was reversed completely. The same phenomenon is seen in figure 4.2 (left side) where spectral changes between 500 and 600 mμ are presented after addition of alkali and back titration with acid. It is concluded from these experiments that $\varepsilon_{540\ m\mu}$ of hemiglobin cyanide is not affected by previous contact with alkali.

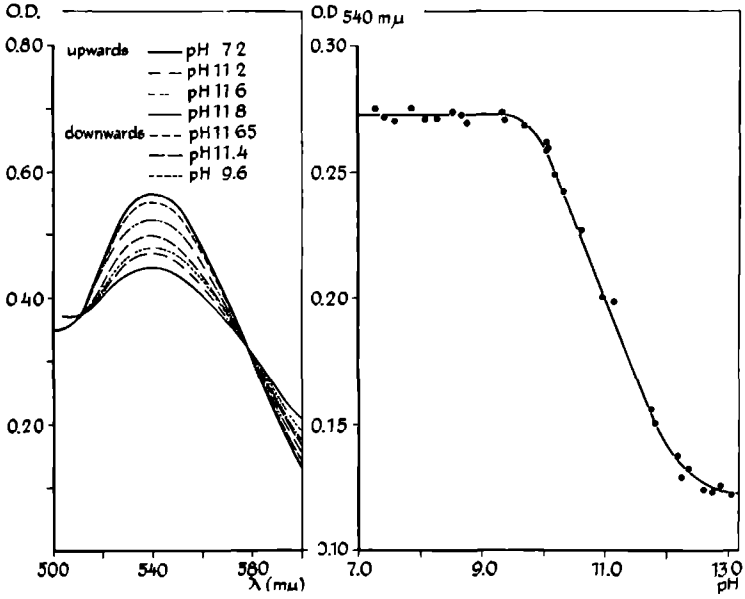


FIGURE 4.2 Hemiglobin cyanide spectra at various pH values (left). Optical density at 540 mμ of a hemiglobin cyanide solution at increasing pH (right).

2. *Alkaline denaturation*

Hemoglobin is known to denature at both ends of the pH scale. Exposure to a pH of 12 as taking place after rapid mixing might induce a conformational change which possibly alters its CO_2 binding properties or interferes with determination of concentration.

Denaturation of hemoglobin was studied by measuring its solubility in ammonium sulfate after previous contact with alkali (Haurowitz, 1934; Brinkman and Jonxis, 1936). Bovine hemoglobin solution was diluted with an equal volume of 0.5 N KOH. After various intervals ranging from 1 to 60 minutes 0.5 ml of this mixture was added to 8 ml of an ammonium sulfate solution in phosphate buffer (500 ml saturated ammonium sulfate in 500 ml 0.15 N Na_2HPO_4 and 55 ml N KH_2PO_4). The solution was centrifuged for 15 minutes and hemoglobin concentration was determined in the supernatant. Experiments were carried out on hemoglobin solutions at room temperature and at 4°C, and on double-blocked carboxyhemoglobin at 4°C.

Any denatured hemoglobin will precipitate under these conditions; its amount may be estimated from the difference between the concentration of the original hemoglobin solution and that of the supernatant after correction for dilution. In figure 4.3 the percentage of native hemoglobin is plotted against time. At

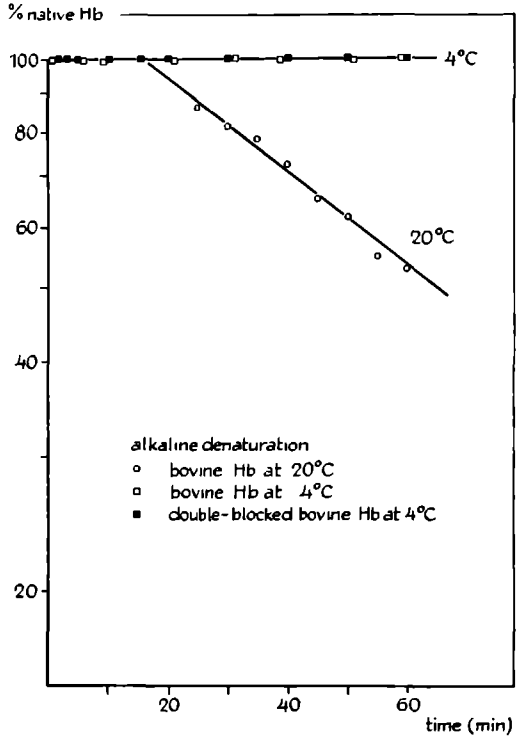


FIGURE 4.3 Alkaline denaturation of bovine hemoglobin (4°C, 20°C) and its double-blocked carbamylated derivative (4°C).

4°C, the same temperature as used in the carbamate experiments, no precipitation is observed, neither for hemoglobin nor for its double-blocked derivative. Besides, hemoglobin concentration of these alkaline solutions, being half of the initial concentration, is found to remain constant for at least 2 hours. The visible and ultraviolet spectra of these hemoglobin solutions in phosphate buffer (pH = 7.40) after various times of exposure to alkali were identical to those of a hemoglobin solution of the same concentration not exposed to alkali. At room temperature some denaturation occurs proceeding according to first order kinetics.

3. *Controls on the separation procedure*

Since it was intended to study the carbamate equilibrium up to a pH value of about 8.4 (at higher pH values bovine hemoglobin will precipitate when equilibrated with CO_2) control experiments were carried out as described by Rossi-Bernardi *et al.* (1969).

CO_2 free hemoglobin solution was mixed with 0.2 M K_2CO_3 in 0.5 N KOH and then anaerobically applied to the column. Complete separation was obtained when a column of 2.5 by 45 cm with a bottom layer of Dower X 10 in OH^-form (2/3 of column length) and Sephadex G 25 C (1/3) at the top was charged with 4 ml of the mixed hemoglobin solution.

When using Dowex some hemoglobin is always retained by the column the amount of which was estimated in the following way. A known quantity of hemoglobin was applied to the column. After elution this quantity was again determined in the pooled fractions. The difference between initial and final quantity of hemoglobin gave the amount retained by Dowex. It was found to be always less than 3 % of the original amount of hemoglobin.

4. *Stabilization of hemoglobin carbamate*

Effect of pH

The efficiency of the alkalinization procedure was studied by performing carbamate determinations on hemoglobin solutions at constant P_{CO_2}, pH and temperature but at varying pH after mixing.

A hemoglobin solution of 9.5 mM/l was equilibrated with a P_{CO_2} of 44 mm Hg at a pH of 7.95 at 37°C and rapidly mixed with KOH of varying strength

(0.2 to 0.5 N). The mixture was applied to the column and eluted with KOH (0.01 to 0.05 N) at a pH value equal to that reached after rapid mixing. In figure 4.4 the number of moles CO_2 bound per mole hemoglobin (Z) as deter-

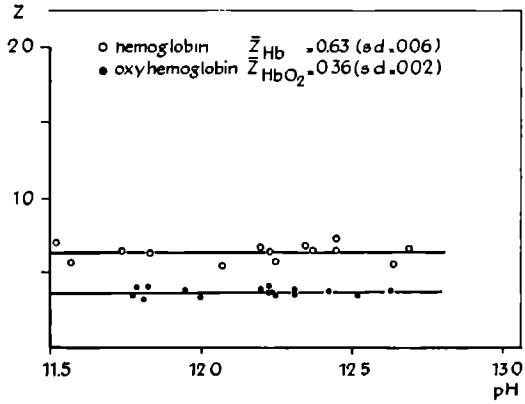


FIGURE 4.4 Number of moles CO_2 bound per mole hemoglobin (Z) equilibrated with a P_{CO_2} of 44 mm Hg (pH 7.95; 37.0°C) at varying pH after mixing.

mined from the effluent and corrected for dissolved CO_2 (see next section) is plotted against the pH after mixing for both hemoglobin and oxyhemoglobin. Regression lines ($Z_{Hb} = 0.55 + 6.79\ 10^{-3}$ pH and $Z_{HbO2} = 0.35 + 1.25\ 10^{-3}$ pH) are parallel to the abscissa within experimental errors, indicating that pH after mixing is sufficiently high to prevent any pH-dependent decomposition of carbamate initially present in solution.

Effect of time

The effect of time on the decomposition of carbamate was studied by determining the carbamate yield at varying intervals of time after mixing. The hemoglobin solution equilibrated with a P_{CO_2} of 44 mm Hg and brought to a pH of 12.0 was applied to the column and left on top of the resin for 0, 5, 10, 20 and 30 minutes before elution was started. All CO_2 arising from decomposed carbamate will immediately turn into carbonate which then will be retained by the column. Any dissociation of carbamate will therefore reveal itself in giving lower carbamate yields at increasing intervals of time. Evaluation by linear regression shows that there is, both for hemoglobin and oxyhemoglobin, a

decrease of Z with time ($\Delta Z_{Hb}/\Delta t = -1.2\ 10^{-3}$ and $\Delta Z_{HbO_2}/\Delta t = -0.7\ 10^{-3}$; t in minutes) which however is small enough (after 10 min less than 3 % of the initial Z value, compared with the experimental error of Z of 5–10 % for Z values of 0.3 and higher) to be neglected as long as elution time remains within 10 minutes.

5. *Blank correction for dissolved CO_2*

During the alkalinization procedure and before separation is started, physically dissolved CO_2 will partly turn into carbonate and partly into carbamate. That part which passed into carbonate will be retained by the column, the other part of newly formed carbamate, however, will add to the carbamate concentration originally present in solution. The final amount of carbamate must be corrected for this extra carbamate resulting from dissolved CO_2. This may be done by performing blank experiments on hemoglobin solutions of the same concentration and equilibrated with the same CO_2 partial pressure as in the actual experiments but at a pH value low enough to prevent carbamate formation. Appropriate values for pH after equilibration range from 6.0 to 6.4. Lower values are known to disturb the dimer-tetramer equilibrium of bovine hemoglobin (Hanlon *et al.*, 1971) and might affect its CO_2 binding properties. The carbamate yield Z as measured in a blank experiment is entirely due to dissolved CO_2 and must therefore be subtracted from the Z values obtained in similar experiments at neutral and alkaline pH. For instance with a hemoglobin solution (11.6 $\pm$ 0.1 mM (Fe)/l) equilibrated with a P_{CO_2} of 45 $\pm$ 1 mm Hg, a Z value of 0.08 $\pm$ 0.02 is obtained due to dissolved CO_2 (pH after mixing is 12.20).

6. *Carbamate equilibrium of hemoglobin and oxyhemoglobin at constant P_{CO_2} and varying pH*

Results of carbamate experiments on hemoglobin and oxyhemoglobin at 37.0° C are shown in figure 4.5. The number of moles CO_2 bound per mole of hemoglobin (Z) is plotted against the pH of the initial hemoglobin solution after equilibration with CO_2 (44 mm Hg). In these conditions carbamate formation starts, to a measurable extent, at pH 6.8. The difference in bound CO_2 between reduced and oxyhemoglobin clearly demonstrates the oxylabile nature of the CO_2 binding properties of hemoglobin at physiological and higher pH values.

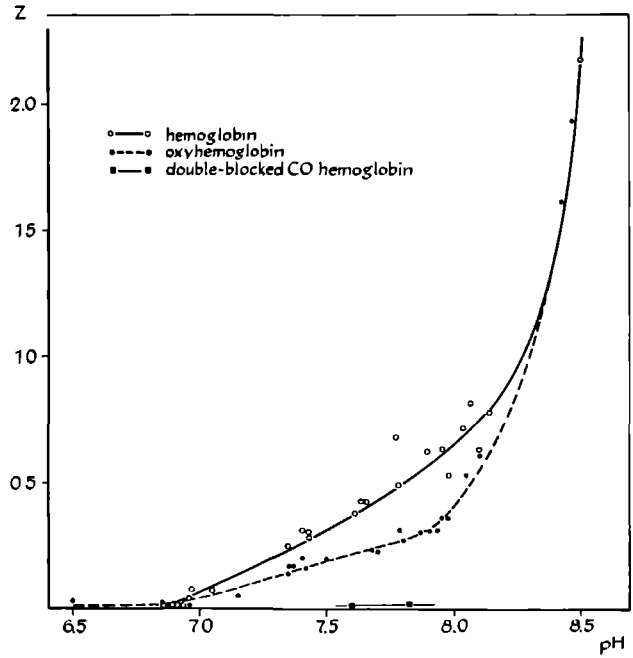


FIGURE 4.5 Plot of Z against pH after equilibration of bovine hemoglobin, oxyhemoglobin and double-blocked carboxyhemoglobin at a P_{CO_2} of 44 mm Hg. Temperature is 37.0°C.

At a pH of about 8.2 the value of Z for hemoglobin exceeds 1 due to the fact that, besides the binding sites in the physiological range which are supposed to be terminal amino groups (Kilmartin and Rossi-Bernardi 1969), other residues become available for CO_2 binding (ε amino groups).

Carbamate determinations performed on carboxyhemoglobin blocked at the terminal amino groups of both α and β chains failed to show any carbamate formation at all. This is a final proof for the exclusive role of the terminal amino groups of hemoglobin in CO_2 binding under physiological conditions of pH and P_{CO_2}.

In order to determine whether the difference in Z between hemoglobin and oxyhemoglobin can fully account for the difference in CO_2 content as observed between hemoglobin and oxyhemoglobin when in total equilibrium with CO_2, estimations of CO_2 content and carbamate determinations were performed on the same hemoglobin solution of 8.6 mM/l equilibrated with a P_{CO_2} of 40 mm Hg at constant temperature (37.0°C) and ionic strength (0.18) but at varying pH. Figures 4.6.a, b gives a plot of the CO_2 content at various pH values. In

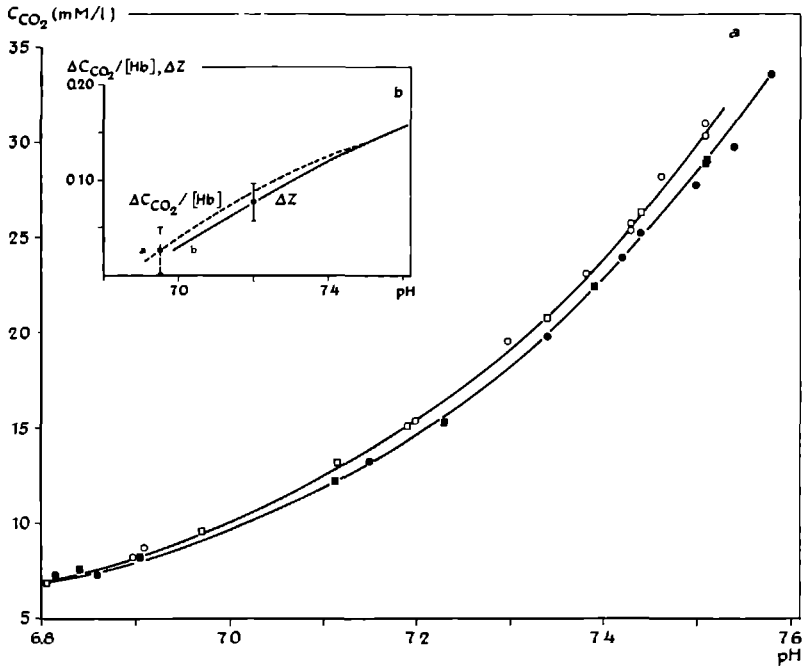


FIGURE 4.6

a Relation between CO_2 content (C_{CO_2}) and pH for hemoglobin and oxyhemoglobin solutions in equilibrium with a P_{CO_2} of 40 mm Hg. Hemoglobin concentration is 8.6 mM (Fe)/l, ionic strength 0.18 and temperature 37.0°C. Symbols (○, ●) refer to experiments where C_{CO_2} and Z were determined on the same hemoglobin sample, whereas (□, ■) indicate C_{CO_2} estimations only, (○, □: hemoglobin; ●,■: oxyhemoglobin)

b Difference in C_{CO_2} (ΔC_{CO_2}) per mole hemoglobin and difference in Z (ΔZ) between hemoglobin and oxyhemoglobin at a certain pH, plotted against pH after equilibration with CO_2 (P_{CO_2} is 40 mm Hg). C_{CO_2} and Z values were obtained from the same hemoglobin solution. Standard deviation is indicated by vertical bars.

figure 4.6b the difference in CO_2 content at each pH value divided by the hemoglobin concentration is plotted against pH, together with the difference in Z obtained from carbamate determinations on the same hemoglobin solution. Standard deviations are indicated by vertical bars. It is concluded that within limits of error the extra carbamate bound to hemoglobin fully covers the difference in CO_2 content between hemoglobin and oxyhemoglobin in total equilibrium with CO_2. At least at this level of accuracy no other forms of CO_2 bound hemoglobin (i.e. y-bound CO_2) need to be taken into account. Therefore it is permitted to calculate the first apparent dissociation constant of carbonic

acid in concentrated hemoglobin solutions from CO_2 content (C_{CO_2}) and carbamate-bound CO_2 according to a modified form of the Henderson-Hasselbalch equation:

$$pH = pK_1^{*\prime} + \log \frac{C_{CO_2} - S \,.\, P_{CO_2} - Z\,[Hb]}{S.P_{CO_2}} \qquad (1)$$

where S is the solubility of CO_2 in mM/mm Hg (0.0321 at 37°) and [Hb] the hemoglobin concentration in mM (Fe)/l.

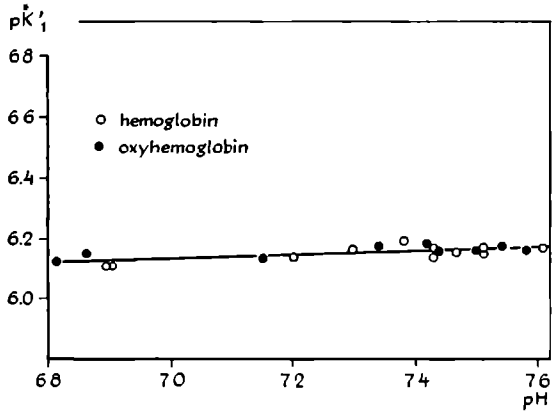


FIGURE 4.7 Relation between apparent first dissociation constant of carbonic acid ($K_1^{*\prime}$) and pH of hemoglobin and oxyhemoglobin solutions equilibrated with a P_{CO_2} of 40 mm Hg at 37.0°C.

Figure 4.7 shows $pK_1^{*\prime}$ values both for hemoglobin and oxyhemoglobin solutions of constant concentration (8.47 ± 0.04) and at constant ionic strength (0.18) but under varying conditions of pH (6.8 – 7.6). Linear regression analysis gives for hemoglobin $pK_1^{*\prime}{}_{Hb} = 5.87 + 0.040$ pH and for oxyhemoglobin $pK_1^{*\prime}{}_{HbO_2} = 5.50 + 0.090$ pH. The deviation of the intercept and slope values is for hemoglobin 0.20 and 0.027 respectively and for oxyhemoglobin 0.15 and 0.021 respectively.

In combining both regression lines which were shown to differ not significantly from each other, a $pK_1^{*\prime}$ of 5.71 (± 0.13) + 0.061 (± 0.017) pH was obtained. When applying this experimental apparent pK value to hemoglobin solutions in total equilibrium with CO_2 and under the same circumstances of temperature,

ionic strength, and hemoglobin concentration as mentioned above, carbamate concentration may in turn be derived from measurements of pH, P_{CO_2} and CO_2 content only.

7. *Carbamate equilibrium of hemoglobin and oxyhemoglobin at constant pH and varying* P_{CO_2}

Hemoglobin and oxyhemoglobin solutions were equilibrated with various CO_2 concentrations (P_{CO_2} ranging from 0–120 mm Hg). At each CO_2 concentration at least 3 equilibrations were performed (each at a different pH value but all within a pH range of 7.35–7.45). Carbamate concentrations estimated by the column method were determined at a pH of 7.40 by linear interpolation of data obtained at different pH values. In figure 4.8 Z values are plotted against P_{CO_2}

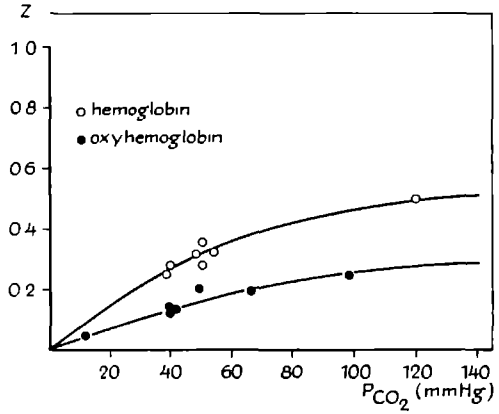


FIGURE 4.8 CO_2 binding curves for hemoglobin and oxyhemoglobin solutions equilibrated with various CO_2 concentrations at a constant pH of 7.40. Temperature is 37.0°C.

at a constant pH value of 7.40 both for hemoglobin and oxyhemoglobin. Again the oxylabile nature of carbamate formation is clearly seen. The hyperbolic binding curves obtained in this way are true CO_2 equilibrium curves analogous to the oxygen equilibrium curve. Similar CO_2 equilibrium curves as presented here have been published recently (Perrella *et al.*, 1971).

8. *Effect of 2,3-diphosphoglycerate on carbamate formation*

Apart from the well known effect on oxygen equilibrium (Benesch and Benesch, 1967; Chanutin and Curnish, 1967) 2,3-diphosphoglycerate (2,3-DPG) also influences the affinity of hemoglobin for CO_2 (Bauer, 1969). The antagonistic nature of this effect has been demonstrated in hemoglobin solutions of various species (Tomita and Riggs, 1971) with the exception of bovine hemoglobin. Estimations of CO_2 content at constant P_{CO_2}, ionic strength, and hemoglobin concentration but at varying pH have been carried out with and without addition of 0.5 mole 2,3-DPG per mole (Fe) hemoglobin. Bovine hemolysates are relatively free of 2,3-DPG (Rapoport, 1969). No special treatment is therefore needed to remove it from hemoglobin solutions. The use of phosphate buffers however has been avoided in view of their effect on the hemoglobin oxygen equilibrium.

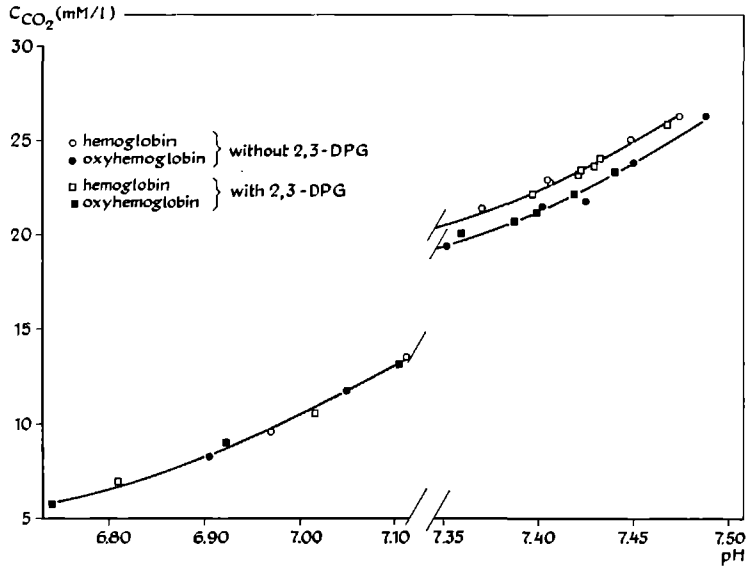


FIGURE 4.9 Relation between CO_2 content and pH for hemoglobin and oxyhemoglobin solutions in equilibrium with CO_2 (P_{CO_2} 40 mm Hg) with and without addition of 2,3- DPG (0.5 mole per mole (Fe) hemoglobin).

Results are shown in figure 4.9. No difference in CO_2 content could be demonstrated between solutions with and without 2,3-DPG, neither for hemoglobin nor for oxyhemoglobin.

In preliminary experiments it could also be shown that the oxygen equilibrium curve of bovine hemoglobin was not displaced upon addition of 2,3-DPG at constant pH, or to a very slight extent only.
This suggests that there is no functional interaction between hemoglobin and 2,3-DPG or probably no binding at all.

DISCUSSION

1. *Bovine hemoglobin at alkaline pH*

Although it has been shown (van Kampen and Zijlstra, 1961) that concentrations of hemoglobin as hemiglobin cyanide may be determined without any difficulty up to a pH of 9, it is unknown what will be the effect of still higher pH values as met after rapid mixing and elution.
The change in O.D. at 540 mμ observed after addition of alkali is completely reversed upon back-titration with acid (Figure 4.2). Similar spectral changes were demonstrated in the cyanmet derivatives of human hemoglobin and horse myoglobin, which were all completely reversible upon acidification. It should be noted that the decrease in O.D. at 540 mμ by alkali is not due to formation of hemiglobin hydroxide. From the ratio of O.D. at pH 13 and at pH 9 a millimolar extinction coefficient of about 5 is obtained for this alkaline hemoglobin derivative in contrast to an ε of 9.7 for hemiglobin hydroxide as reported by Lemberg and Legge (1949). Besides, its spectrum is different from that of hemiglobin hydroxide. Reversible hemichrome formation seems more likely to be involved (Rieder, 1970). The millimolar extinction coefficient is of the same order of magnitude as that of hemichrome derivatives and its spectrum is similar, at least qualitatively, to hemichrome spectra (Falbe-Hansen, 1961).
In addition to hemiglobin cyanide, hemiglobin as well as carboxyhemoglobin appear to be insensitive to a previous exposure for one hour to a pH of 12 (4°C), which makes possible the application of the stabilization procedure.

2. *Methodical considerations in carbamate estimation*

In the physiological range of P_{CO_2} and pH, CO_2 is supposed to react reversibly with hemoglobin at its terminal amino groups forming carbamino-hemoglobin (Henriques, 1928; Ferguson and Roughton, 1934; Stadie and O'Brien, 1937; Rossi-Bernardi and Roughton, 1967; Kilmartin and Rossi-Bernardi, 1969).

This carbamate reaction only takes place with uncharged amino groups (Faurholt, 1925). Using symbols introduced by Roughton (1964) carbamate equilibrium may be described by the following reactions:

$$HbNH_3^+ \rightleftharpoons HbNH_2 + H^+ \qquad K_z \qquad (2)$$

$$HbNH_2 + CO_2 \rightleftharpoons HbNHCOOH \qquad K \qquad (3)$$

$$HbNHCOOH \rightleftharpoons HbNHCOO^- + H^+ \qquad K_x \qquad (4)$$

where Hb refers to one hemoglobin chain with a single terminal amino group and K, K_x and K_z are the equilibrium constants of the respective reactions. Reactions (3) and (4) may be combined by introducing an apparent equilibrium constant K_c which equals $K.K_x$. Carbamate concentration [HbNHCOOH] + + [HbNHCOO$^-$] may then be obtained from:

$$[\text{carbamate}] = \frac{1}{1 + \frac{[H^+]}{K_c[CO_2]} + \frac{[H^+]^2}{K_zK_c[CO_2]}} [\text{amine}]_{\text{total}} \qquad (5)$$

where $[\text{amine}]_{\text{total}}$ is the total concentration of terminal amino residues in whatever form present in solution ($HbNH_3^+$, $HbNH_2$, HbNHCOOH and HbNHCOO$^-$), equalling the hemoglobin concentration expressed in moles Fe/l. Taking for K_c and K_z the approximate values for bovine hemoglobin at 37.0°C given in table 4.1, a hemoglobin solution of 10 mM(Fe)/l equilibrated at 37°C with a P_{CO_2} of 44 mm Hg at a final pH of 7.40 would contain about 3 mM/l carbamate. To determine the carbamate concentration experimentally these 3 mM/l bound CO_2 should be separated from about 25 mM/l CO_2 present as dissolved CO_2 and bicarbonate.

Disturbances of equilibrium conditions will change carbamate concentration at a rate of

$$\frac{-d[HbNHCOOH]}{dt} = k_d \frac{[HbNHCOO^-]\,[H^+]}{K_x} - k_a[CO_2]\,[HbNH_2] \qquad (6)$$

k_a and k_d being the velocity constants of the association and dissociation reaction of (3). Approximate values of k_a, k_d and K_x are given in table 4.1.

TABLE 4.1 Approximate values of equilibrium and velocity constants related to carbamate formation and CO_2 water equilibrium.

	Value	Temp.	Compound	Reference
K_z	$10^{-7.58}$	37.0°C	hemoglobin	Rossi-Bernardi and Roughton, 1967, from data of Stadie and O.Brien, 1937
K_c	$10^{-4.53}$	37.0°C	hemoglobin	idem
	$10^{-4.85}$	20.0°C	hemoglobin	Roughton, 1970
K_x	10^{-5}—10^{-6}	37.0°C	hemoglobin	Roughton, 1970
	$10^{-5.2}$	5.0°C	glycylglycine	Roughton and Rossi-Bernardi, 1970
k_a	1585 M^{-1} sec^{-1}	20.0°C	glycylglycine	Chipperfield, 1966
k_d	410 sec^{-1}	20.0°C		calculated from k_a, K_c and K_x = $10^{-5.45}$
K_1'	$10^{-6.381}$	20.0°C		Harned and Davis, 1943
K_2	$10^{-10.377}$	20.0°C		Harned and Scholes, 1941
k_1	5900 $M^{-1}sec^{-1}$	20.0°C		Pinsent, Pearson and Roughton, 1956
k_2	96.6 10^{-6} sec^{-1}	20.0°C		calculated from k_1, K_1 and K_w

If the hemoglobin solution is immediately applied to a Sephadex-Dowex column and eluted at the same pH of 7.40 it may be computed from (6) that no carbamate will be left after a time delay of 200 seconds needed for elution. Representative results might however be obtained by changing the equilibrium conditions in such a way as to make both terms of the right-hand part of equation (5) as small as possible. This is achieved by raising instantaneously the pH of the hemoglobin solution to about 12. Dissociation of carbamate is impaired by a shift in the equilibrium state of the CO_2 carbamate system which displaces reactions (2) and (3) to the right; formation of fresh carbamate from dissolved CO_2 is at least partially prevented by accompanying alterations in the equilibrium state of the CO_2-bicarbonate-carbonate system.

In evaluating the kinetics of both systems after a sudden rise of pH the following reactions should be considered too:

$$CO_2 + OH^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HCO_3^- \tag{7}$$

$$HCO_3^- + OH^- \rightleftharpoons CO_3^{=} + H_2O \tag{8}$$

Dissolved CO_2 will decrease at a rate:

$$\frac{-d[CO_2]}{dt} = k_1[CO_2]\,[OH^-] + k_a[CO_2]\,[HbNH_2] - k_2[HCO_3^-] \qquad (9)$$

$$- k_d[HbNHCOOH]$$

$[CO_2]_{total}$ is defined as:

$$[CO_2]_{total} = [CO_2] - [HCO_3^-] + [CO_3^=] + [HbNHCOOH] + [HbNHCOO^-] \qquad (10)$$

Since at pH 12 equilibrium of (3) and (7) is reached within a few milliseconds or less (Eigen and de Maeyer, 1963), i.e. much faster than in (2) and (6), equation (9) becomes:

$$[CO_2]_{total} = [CO_2] + [CO_3^=]\left(\frac{[H^-]}{K_2} + 1\right) + [HbNHCOO^-]\left(\frac{[H^+]}{K_x} + 1\right) \qquad (11)$$

where K_2 is the second equilibrium constant of the CO_2 - HCO_3^- - $CO_3^=$ system.

$$\text{At a pH value of 12 } \frac{[H^+]}{K_2} \ll 1 \text{ and } \frac{[H^+]}{K_x} \ll 1, \text{ so} \qquad (12)$$

$$[CO_3^=] = [CO_2]_{total} - [CO_2] - [HbNHCOO^-]$$

After substitution and rearrangement (9) may be written as

$$\frac{-d[CO_2]}{dt} = [CO_2]\left(k_1[OH^-] + k_a[HbNH_2] + \frac{[H^+]}{K_2}k_2\right) - \qquad (13)$$

$$[H^+]\left\{\frac{k_2}{K_2}[CO_2]_{total} - \left(\frac{k_2}{K_2} - \frac{k_d}{K_x}\right)[HbNHCOO^-]\right\}$$

When applying rapid mixing with a multi-jet mixing chamber the pH of 12 will be attained in a millisecond or so, i.e., in about the same time as needed for buffer reactions at protein residues to be complete. Under these circum-

stances equation (13) is a differential equation of the type $-\dot{x} = ax - b$ where a and b may be considered to be constant for a time interval of at least 500 sec after mixing. This equation may be solved for:

$x = \frac{b}{a} + \left(x_0 - \frac{b}{a}\right) e^{-at}$ where x_0 is the concentration of dissolved CO_2 at $t = 0$,

i.e., half the concentration of dissolved CO_2 before mixing. On substitution of numerical values (table 4.1) for rate and equilibrium constants at 20°C, i.e. the temperature of the hemoglobin solution immediately after mixing, and taking for total CO_2, carbamate and $HbNH_2$ (including 12 ε amino groups per chain) half of the original concentrations, equation (13) becomes

$$CO_2 = 14.10^{-10} + 66.10^{-5}\, e^{-143t} \tag{14}$$

It follows that a quasi-equilibrium state up to 99 % will be reached within 120 m sec after mixing, a time interval too short for the dissociation reaction to proceed to a measurable extent.

The concentration of carbamate freshly formed from dissolved CO_2 may be estimated from the rate equations of reactions (2) and (6). The ratio Q of dissolved CO_2 turning into carbamate and carbonate is given by

$$Q = \frac{k_a[CO_2]\,[HbNH_2] - \frac{k_d}{K_x}\,[HbNHCOO^-]\,[H^+]}{k_1[CO_2]\,[OH^-] \quad - \frac{k_z}{K_x}\,[H^+]\,[CO_3^=]} \tag{15}$$

When inserting appropriate values from table 4.1, Q becomes 2.5. Hence about 70 % of the dissolved CO_2 is converted to carbamate, i.e., 0.9 mM/l of the dissolved CO_2 initially present after equilibration. At a hemoglobin concentration of 10 mM/l this will raise the Z values by 0.09, which is of the same order of magnitude as the experimentally obtained blank correction of 0.08 ± 0.02 for Z (pH after mixing is 12.2).

Alterations in reaction conditions upon rapid mixing and kinetics of equilibrium displacement in both CO_2-carbamate and CO_2-bicarbonate-carbonate system resulting from this sudden rise of pH are schematically shown in figure 4.10.

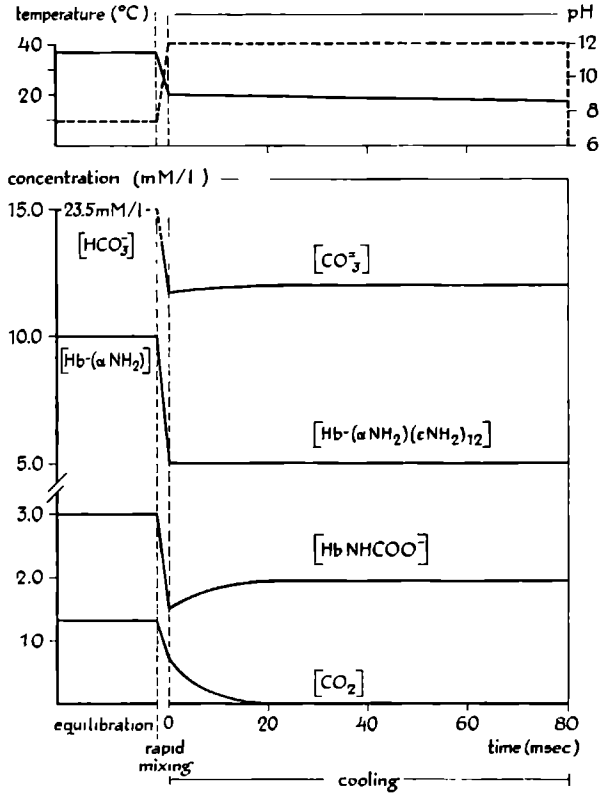


FIGURE 4.10 Kinetics of equilibrium displacement in the CO_2-carbamate and CO_2-bicarbonate-carbonate system after a sudden rise of pH accompanied by a decrease of temperature; (αNH_2), $(\varepsilon NH_2)_{12}$ indicate the species and number of groups available for carbamate formation.

3. *Carbamate equilibrium*

The results shown in figure 4.5 are in reasonable agreement with the scarce data reported by other authors and obtained with different techniques. Under circumstances only slightly different from those in figure 4.5 Ferguson and Roughton (1934) obtained, with the barium precipitation method, for hemoglobin a Z value of 0.23 at a pH of 7.33 (calculated from table II of their paper) compared with 0.24 $\pm$ 0.02 in this study. Stadie and O'Brien, using a total CO_2 equilibrium technique (1937), gave for hemoglobin a Z value of 0.38 at a pH of 7.39 (derived from their table VI) in contrast to a value of 0.27 $\pm$ 0.02 as

shown in figure 4.5. The P_{CO_2} however, was about 4 mm Hg higher in their situation which may, at least partially, explain the observed discrepancy.
The difference in Z (Δ Z) between hemoglobin and oxyhemoglobin indicates the number of moles of extra CO_2 bound to hemoglobin upon complete deoxygeneration of 1 mole of oxyhemoglobin at constant pH. At a pH of 7.40 and a P_{CO_2} of 44 mm Hg Δ Z is equal to 0.11 $\pm$ 0.04, i.e., about half the value found in solutions of human hemoglobin (0.21 obtained by Ferguson (1936) and corrected by Roughton (1970), and 0.23 derived from data of Bauer (1970)). Bovine hemoglobin therefore binds about half the number of moles of CO_2 per mole of oxygen released at constant pH as compared with human hemoglobin.
At a pH $>$ 8 a steep rise in Z is observed (Z values for hemoglobin exceeding 1 at pH 8.2). Since in control experiments the column was shown to separate completely the large quantities of bicarbonate and carbonate present in this situation the increase of Z has to be attributed exclusively to carbamate formation at ε amino groups becoming available for CO_2 binding at these higher pH values. Equation (5) is therefore modified in such a way as to include also the contribution of the ε amino groups. When adopting the equilibrium equations (2,3 and 4) carbamate concentration becomes

$$[\text{Carbamate}] = \sum_{j=0}^{n} \sum_{i=0}^{n-j} (j+1)\,[\text{Hb } a^c\,(e^c)^j\,e^i\,(e^+)^{n-i-j}] \qquad (16)$$

and hemoglobin concentration

$$[\text{Hb}] = \sum_{j=0}^{n} \sum_{i=0}^{n-j} [\text{Hb } a^c(e^c)^j\,e^i\,(e^+)^{n-i-j}] + \qquad (17)$$

$$\sum_{k=0}^{n} [\text{Hb a } e^k(e^+)^{n-k}] + [\text{Hb } a^+(e^+)^n]$$

Hb a, Hb a^+, Hb a^c refer to the concentrations of neutral, positively charged and carbamylated α amino groups and Hb e, Hb e^+, Hb e^c to the concentrations of ε amino groups in their corresponding state. When inserting equilibrium constants $K_{ca,e}$ and $K_{za,e}$ for both groups

$$[\mathrm{Hb\ a^c(e^c)^j\ e^i\ (e^+)^{n-i-j}}] = \left(\frac{K_{ce}\,[CO_2]}{[H^+]}\right)^j \cdot \frac{K_{ca}[CO_2]}{[H^+]} \cdot \left(\frac{K_{ze}}{[H^+]}\right)^{(i+j)} \cdot \frac{K_{za}}{[H^+]}\,[\mathrm{Hb\ a^+(e^-)^n}] \tag{18}$$

$$[\mathrm{Hb\ a\ e^k(e^+)^{n-k}}] = \left(\frac{K_{ze}}{[H^+]}\right)^k \frac{K_{za}}{[H^+]}\,[\mathrm{Hb\ a^+(e^+)^n}] \tag{19}$$

Z is obtained by taking the quotient of (16) and (17) after substitution of (18) and (19); n is taken to be 12 which is the number of ε amino groups per chain of bovine hemoglobin (Dayhoff, 1969), probably all titratable and capable of CO_2 binding.

It was attempted to calculate, from the data of figure 4.5, K_z and K_c values for both α and ε amino groups. Data up to a pH of 7.6 were inserted into equation (20), a variant of equation (5):

$$\frac{[CO_2]}{[H^+]}\left(\frac{1-Z}{Z}\right) = \frac{1}{K_c} + \frac{[H^+]}{K_zK_c} \tag{20}$$

Plotting the left side of equation (20) against $[H^+]$ and applying least square methods failed to give the linear relationship previously obtained by Rossi-Bernardi and Roughton (1967) from data of Ferguson (5 experimental points only). Using a polynomal approximation procedure where data were fed into a computer and fitted according to $1/Z = A_0 + A_1[H^+] + A_2[H^+]^2 + A_3[H^+]^3$ and $1/Z = A_0 + A_1[H^+]^2 + A_2[H^+] + A_3[H^+]^{-1}$, derived from equations (5) and (16, 17) respectively, gave values for K_c ($pK_{caHb} \sim 4.8$, $pK_{caHbO_2} \sim 5.1$, $pK_{ce} \sim 5.5$) but failed to provide reliable K_z values (negative values in the case of oxyhemoglobin).

According to common practice (van Kempen and Kreuzer, 1971; Perrella *et al.*, 1971) it is implicitly assumed in the equilibrium reactions (2) to (5) that terminal amino groups of both α and β chains of hemoglobin are equally involved in carbamate formation.

The asymmetric functional behavior of these residues in Bohr effect and 2,3-DPG binding, where K_z values are involved in both cases, makes this assumption highly improbable.

A more refined analysis on the basis of non-equivalent but independent binding sites may be given in relation to data shown in figure 4.8. Within the tetrameric molecule these binding sites are supposed to combine with CO_2 independently, each with its own association constant according to:

$$\begin{aligned} Hb + CO_2 &\rightleftharpoons HbCO_{2\alpha 1} \qquad K_{\alpha 1} \\ Hb + CO_2 &\rightleftharpoons HnCO_{2\alpha 2} \qquad K_{\alpha 2} \\ Hb + CO_2 &\rightleftharpoons HbCO_{2\beta 1} \qquad K_{\beta 1} \\ Hb + CO_2 &\rightleftharpoons HbCO_{2\beta 2} \qquad K_{\beta 2} \end{aligned} \tag{21}$$

where $\alpha_{1,2}$ refers to binding sites on both α chains and $\beta_{1,2}$ to those on the β chains. A second CO_2 molecule may be bound to each of three binding sites left, for instance to the β_1 amino group, according to

$$HbCO_{2\alpha 1} + CO_2 \rightleftharpoons HbCO_{2\alpha 1} \,.\, CO_{2\beta 1} \qquad K_{\beta 1} \tag{22}$$

Treating similarly the combination with a third and fourth CO_2 molecule, total concentration of CO_2 bound to hemoglobin becomes:

$$[\text{Carbamate}] = P[CO_2] + 2Q[CO_2]^2 + 3R[CO_2]^3 + 4S[CO_2]^4 \tag{23}$$

and hemoglobin concentration:

$$[Hb] = 4(1 + P[CO_2] + Q[CO_2]^2 + R[CO_2]^3 + S[CO_2]^4) \tag{24}$$

where
$$P = K_{\alpha 1} + K_{\alpha 2} + K_{\beta 1} + K_{\beta 2} \tag{25}$$
$$Q = K_{\alpha 1} K_{\alpha 2} + K_{\beta 1} K_{\beta 2} + K_{\alpha 1} K_{\beta 1} + K_{\alpha 1} K_{\beta 2} + K_{\alpha 2} K_{\beta 1} + K_{\alpha 2} K_{\beta 2}$$
$$R = K_{\alpha 1} K_{\alpha 2} K_{\beta 1} + K_{\alpha 1} K_{\beta 1} K_{\beta 2} + K_{\alpha 1} K_{\beta 1} K_{\beta 2} + K_{\alpha 2} K_{\beta 1} K_{\beta 2}$$
$$S = K_{\alpha 1} K_{\alpha 2} K_{\beta 1} K_{\beta 2}$$

Z is obtained from the quotient of equations (23) and (24).
If for reasons of structural symmetry of hemoglobin, binding sites on identical chains are supposed to be equivalent ($K_{\alpha 1} = K_{\alpha 2} \simeq K_\alpha$ and $K_{\beta 1} = K_{\beta 2} \simeq K_\beta$) Z simplifies to:

$$Z = \tfrac{1}{2} \frac{K_\alpha[CO_2]}{1 + K_\alpha[CO_2]} + \tfrac{1}{2} \frac{K_\beta[CO_2]}{1 + K_\beta[CO_2]} \tag{26}$$

Combining equations (26) and (5) relates the association constants K_α and K_β to their corresponding K_c and K_z constants:

$$K_{\alpha,\beta} = \frac{K_{z\alpha,\beta} \cdot K_{c\alpha,\beta}}{K_{z\alpha,\beta}\,[H^+] + [H^+]^2} \tag{27}$$

Equation (26) is rearranged to

$$Z = (K_\alpha + K_\beta)\,(\tfrac{1}{2} - Z)\,[CO_2] + K_\alpha \cdot K_\beta\,(1 - Z)\,[CO_2]^2 \tag{28}$$

and the data shown in figure 4.8 were found to fit this equation for K_α and K_β values of $1.53\ 10^{-2}$ and $0.46\ 10^{-2}$ in the case of hemoglobin and $7.7\ 10^{-3}$ and $0.65\ 10^{-3}$ in the case of oxyhemoglobin.
The error of $(K_\alpha + K_\beta)$ and $K_\alpha \cdot K_\beta$ was $0.24\ 10^{-2}$ and $6.9\ 10^{-6}$ for hemoglobin, and $0.024\ 10^{-2}$ and $1.2\ 10^{-6}$ for oxyhemoglobin.
$K_{\alpha,\beta}$ values are expressed in mm Hg^{-1}. When K_c and K_z values of table 4.1 are inserted into equation (27) $K_{\alpha,\beta}$ values $(M/l)^{-1}$ are obtained which are of the same order of magnitude as the values mentioned above if expressed in the same units.
From the rather few data of figure 4.8 and under the condition made in deriving equation (26) it is concluded that terminal amino groups of both α and β chain contribute significantly but not equally to CO_2 binding in hemoglobin as well as in oxyhemoglobin solutions.
K_c and K_z values for individual binding sites may be obtained by solving equation (27) for a similar set of CO_2-binding data taken at a different pH value.
More values at a broader range of P_{CO2} are needed first in order to confirm equation (26) and reduce the error of $K_{\alpha,\beta}$.

4. *Carbamate and bicarbonate in equilibrium with CO_2*

a. The existence of y-bound CO_2

Apart from carbamate formation another type of CO_2 binding has been proposed by several authors. Bicarbonate was shown to displace the oxyhemoglobin dissociation curve, which brought Sidwell *et al.* (1937) and Hermann *et al.* (1939) to postulate a compound formation between hemoglobin and bicarbonate. Arguments of Margaria (1957) in favor of this so-called y-bound CO_2

(Ferguson and Roughton, 1934), were mainly based on the observed discrepancy in carbamate yield obtained with the barium precipitation technique and the total CO_2 equilibrium method. At pH < 7 where carbamate formation is supposed to be negligible the second method yielded appreciable amounts of bound CO_2 which could not be demonstrated by the first method. CO_2 binding at acid pH could not be confirmed by Stadie and O'Brien (1937). Recently Kreuzer *et al.* (1971), adopting a kinetic method, showed that there exists, even at physiological pH, a very slight but definite specific effect of bicarbonate on the affinity of hemoglobin for oxygen.

In the experiments of Milla, Giustina and Margaria (1953) concentration of bound CO_2 was obtained from total CO_2 content by subtracting concentrations of dissolved CO_2 estimated from P_{CO2} in the gas phase and bicarbonate calculated from the Henderson-Hasselbalch equation using the apparent first dissociation constant of carbonic acid (K'_1, where $pK'_1 = 6.33 - 0.54\sqrt{\mu}$ at 37.0°C; Hastings and Sendroy, 1925). In these conditions the dissociation constant of carbonic acid (denoted by $K_1^{*\prime}$) is a purely experimental quantity not determinable by Hastings' equation. $K_1^{*\prime}$ might be expected and has actually been shown to vary with pH in aqueous as well as in protein containing solutions (Siggaard-Andersen, 1962). In human plasma it is found to decrease with increasing pH ($d\ pK_1^{*\prime}/d\ pH = 0.049$ at pH = 7.4; Rispens *et al.*, 1968), in hemoglobin solutions its relation to pH and protein concentration is unknown at present. As far as $K_1^{*\prime}$ values of hemoglobin and oxyhemoglobin in the same conditions of ionic strength, pH and protein concentration are considered to be equal, ΔZ is independant of the $pK_1^{*\prime}$ value chosen. Comparing ΔZ values (rather than Z) obtained by both methods failed to reveal the discrepancy observed by Margaria (figure 4.5b). Therefore any oxylabile y-binding will be limited to the range of error inherent to both methods (error in ΔZ is at least $\pm$ 0.02), which leaves the subject still somewhat controversial.

b. Calculation of $pK_1^{*\prime}$ in hemoglobin solutions

As stated above the first dissociation constant (K_1) of carbonic acid cannot be determined in hemoglobin solutions. It involves knowledge of the activity coefficients of both HCO_3^- and CO_2 which cannot be derived from the equations of Stadie and Hawes (1928) since these equations are obtained from experiments disregarding carbamate formation. It may be attempted to define an entirely experimental apparent dissociation constant ($K_1^{*\prime}$) in hemoglobin solutions.

Under the experimental conditions defined in section 6 of Results, this $pK_1^{*\prime}$ equals 6.159 for both hemoglobin and oxyhemoglobin in agreement with values reported for bovine hemoglobin (6.13 ± 0.05 for Hb and 6.17 ± 0.03 for HbO_2; Rossi-Bernardi and Roughton, 1967). This value tallies reasonably well with the value obtained from the double-blocked derivative after being corrected for the difference in protein concentration (Siggaard-Andersen, 1962). This $pK_1^{*\prime}$ value however is of limited importance in the calculation of carbamate concentration from CO_2 estimations. It may only be used under similar conditions as mentioned above and is expected to give merely approximate values. Using the data of figure 4.7 the ratio of carbamate CO_2 to CO_2 content is calculated to vary, with decreasing P_{CO_2}, from 0.09 to 0.13 for hemoglobin and from 0.04 to 0.08 for oxyhemoglobin (pH 7.4, P_{CO_2} between 20 and 100 mm Hg). Considering the error in the van Slyke technique it follows that carbamate concentration estimated from CO_2 content may be uncertain to at least 10 %.

c. Interaction of bovine hemoglobin with 2,3-DPG

The interaction between hemoglobin and 2,3-DPG, which has been demonstrated in hemolysates of various species including man (Benesch and Benesch, 1967; Beetlestone and Irvine, 1970; Tomita and Riggs, 1971) seems absent in the case of bovine hemoglobin. The inhibitory effect on carbamate formation and probably also the influence on the affinity of hemoglobin for oxygen failed to appear upon addition of 2,3-DPG at constant pH, P_{CO_2} and P_{O_2}.

In this respect it seems an interesting coincidence that 2,3-DPG levels in bovine red cells are about 500 times less than in human erythrocytes (Rapoport, 1969). Hemolysates of sheep erythrocytes where hemoglobin is reported to have no affinity for 2,3-DPG (Perutz, 1970) have equally low 2,3-DPG levels.

2,3-DPG is assumed to react, among other residues, with the terminal amino groups of the α chain (Bunn and Briehl, 1970; Perutz, 1970) in competition with CO_2. The absence of such interaction in bovine hemoglobin may be explained in a similar way as in the case of sheep hemoglobin (Perutz, 1970). From the primary structure of bovine hemoglobin (Schroeder *et al.*, 1967) it appears that its β chain is one residue shorter at the amino end compared with the chain of human and horse hemoglobin. This will increase the distance between terminal amino groups of the β chain and lower the association constant for the two phosphate groups of DPG.

In human hemoglobin 2,3-DPG in a concentration of 0.5 mole per mole heme

decreases Δ Z to about 50 % (Pace, Rossi-Bernardi and Roughton, 1970). It follows that bovine hemoglobin is involved in oxylabile CO_2 transport to about the same extent as human hemoglobin in the presence of 2,3-DPG.

SUMMARY

Hemoglobin-bound CO_2 was estimated by a procedure first described by Rossi-Bernardi, Pace, Roughton and van Kempen (1968) in which the carbamate compound is stabilized by rapid mixing with alkali and then separated from other CO_2 constituents in solution by gel filtration and ion exchange chromatography. Z, the number of moles CO_2 bound per mole (Fe) hemoglobin, is obtained from the anaerobically sampled hemoglobin solution by standard gas analysis and hemoglobin concentration determination. The efficiency of the separation procedure is checked in control experiments and the conversion of dissolved CO_2 into freshly formed carbamate is corrected for by blank experiments. Elution times of 10 minutes or less and pH values after mixing ranging from 11.5 to 12.5 were shown to have no effect on carbamate yield. No alkaline denaturation of bovine hemoglobin was observed in conditions existing during carbamate estimation.

Carbamate equilibrium of bovine hemoglobin was studied at constant P_{CO_2} and varying pH as well as at constant pH and varying P_{CO_2} (ionic strength 0.18, temperature 37.0° C). The difference in Z (Δ Z) between hemoglobin and oxyhemoglobin appeared to be 0.11 ± 0.04 (pH = 7.40; P_{CO_2} = 44 mm Hg), i.e. about half the value observed in human hemoglobin. Δ Z was shown to account completely for the difference in CO_2 content (C_{CO_2}) between hemoglobin and oxyhemoglobin when in total equilibrium with CO_2.

Carbamate determinations on bovine hemoglobin specifically modified at all terminal amino groups (double-blocked carbamylated derivative) did not show any CO_2 binding at all, thus giving a final proof for the exclusive role of the terminal amino groups in CO_2 binding under physiological conditions. At pH > 8 Z values exceeded 1, indicating the presence of other residues being available for CO_2 binding (ε amino groups).

Attempts to calculate the ionization constant (K_z) and the carbamate equilibrium constant (K_c) of the terminal amino groups failed, suggesting that not all terminal groups are equivalent in their CO_2 binding properties. This was confirmed by the fact that carbamate data obtained at constant P_{CO_2} and varying pH fitted binding curves derived from two sets of independent but

non-equivalent binding sites. Association constants for both kinds of binding sites appeared to differ by a factor of at least 3 in hemoglobin and of about 10 in oxyhemoglobin.

From determinations of hemoglobin-bound CO_2 and CO_2 content of hemoglobin and oxyhemoglobin solutions in total equilibrium with CO_2, the apparent first dissociation constant of carbonic acid was calculated and found to be independent of the oxygenation state of hemoglobin.

In contrast with hemoglobin of other species bovine hemoglobin appeared to be not influenced by the presence of 2,3-DPG as far as its CO_2 binding properties are concerned. A possible explanation is given from a comparison of the structure of its β-chain with that of human and horse hemoglobin.

Chapter 5

PREPARATION OF BOVINE HEMOGLOBIN DERIVATIVES CARBAMYLATED AT THE TERMINAL AMINO GROUPS

INTRODUCTION

In the preceding chapter terminal amino groups of bovine hemoglobin were shown to be not equally involved in CO_2 binding as has been assumed previously. One of the two association constants related to CO_2 binding sites on α and β chains appeared to be at least 3 times higher in hemoglobin and about 10 times higher in oxyhemoglobin solutions (pH 7.40; 37.0°C). The limited accuracy of data derived from carbamate estimations did not allow further analysis in terms of K_c and K_z values for individual binding sites.

These constants may presumably be determined by studying CO_2 binding properties of hemoglobin derivatives modified at the terminal amino groups of either α or β chain in such a way as to inhibit their reaction with CO_2. One of the main requirements to be met is that modification of one of these binding sites does not affect the functional properties of the other sites. In practice this means that no conformational changes of hemoglobin structure are allowed to occur and that cooperative effects within the protein molecule must remain unchanged.

Hemoglobin is readily modified at its terminal amino groups by treatment with 1-fluoro-2,4-dinitrobenzene (FDNB) (Hill and Davis, 1967). The hemoglobin derivative thus obtained appeared to have lost heme-heme interaction and Bohr effect completely (Neer and Koningsberg, 1968) and its crystallographic structure showed large deviations from that of the native protein (Kilmartin and Rossi-Bernardi, 1969).

Another approach is to study CO_2 binding properties of hemoglobin in the presence of 2,3-DPG which is known to combine reversibly with the terminal amino groups of the β chain (Bunn and Briehl, 1970). Such combination which is observed in hemoglobins of various species does not occur, however, in bovine hemoglobin or only to a slight extent.

Treatment of horse hemoglobin (fast component) with cyanate was shown to block, under certain conditions, specifically the terminal amino groups of α or β chain without inducing any significant conformational change or affecting heme-heme interaction (Kilmartin and Rossi-Bernardi, 1969).

MATERIALS AND METHODS

Preparation of hemoglobin solution.

In view of the previous study on carbamate equilibrium of bovine hemoglobin the cyanate-blocked hemoglobin derivatives were prepared from bovine hemoglobin as well.

Hemoglobin solutions were prepared according to the ether and salt procedure of Adair and Adair (1934) at 4°C from freshly drawn heparinized bovine blood and stored under a positive pressure of carbon monoxide at 4°C. Hemoglobin concentration was determined as hemiglobin cyanide taking into account the much slower conversion time of the carbon monoxide derivative. Methemoglobin concentration in these COhemoglobin solutions was determined according to Kilmartin and Rossi-Bernardi (1971).

Methods used in performing sulfhydryl titration and oxygen equilibrium studies are described under Results.

All reagents used were of analytical grade. Potassium cyanate was obtained from J. T. Baker Chemicals, cystamine dihydrochloride and dithiothreitol from Sigma Chemical Company, glutathione from E. Merck A.G., p-chloromercuribenzoate from Calbiochem, Inc., Sephadex G 25 F from Pharmacia, and Biorex 70 (200–400 mesh Na^+ form) from Bio-Rad Laboratories.

RESULTS

1. *Preparation of carbamylated bovine hemoglobin derivatives*

The procedure was similar to that used by Kilmartin and Rossi-Bernardi (1969, 1971) in preparing carbamylated horse hemoglobin derivatives, except for some modifications in reagent concentrations and reaction conditions.

Cyanate is known to react, except for terminal amino groups, with sulfhydryl groups (-SH groups) as well. Since this reaction appeared to be not completely reversible at alkaline conditions (Kilmartin and Rossi-Bernardi, 1969), at least not in the case of hemoglobin, reactive -SH groups were protected by treatment

with cystamine dihydrochloride (15 moles/mole αβ-dimer, 1 hour at 4.0°C, pH 8.5) before carbamylation was started. The excess of cystamine was removed by gel filtration (Sephadex G 25 F equilibrated with a 0.2 M sodium phosphate buffer of pH 6.20). The cystamine-treated hemoglobin solution was reacted with KCNO (in concentrations varying from 5 to 50 moles/mole αβ-dimer) for one hour at 25.0°C and then passed through a Sephadex G 25 F column equilibrated with 0.025 M/l Na_2HPO_4 to remove the excess of KCNO. The reacted -SH group was freed from cystamine by treatment with dithiothreitol (15 moles/mole αβ-dimer) at 4.0°C and pH 8.5.

After gel filtration (Sephadex G 25 F equilibrated with a 0.05 M sodium phosphate buffer of pH 6.20; Na_2HPO_4 0.94 g/l and $NaH_2PO_4.2\ H_2O$ 5.66 g/l) the hemoglobin solution was applied to a Biorex 70 column previously equilibrated with the same buffer. The column was developed by a continuous linear salt gradient of 0.05 to 0.10 M sodium followed by 0.15 M sodium. The elution pattern for a column of 2 × 40 cm charged with about 2.5 g hemo-

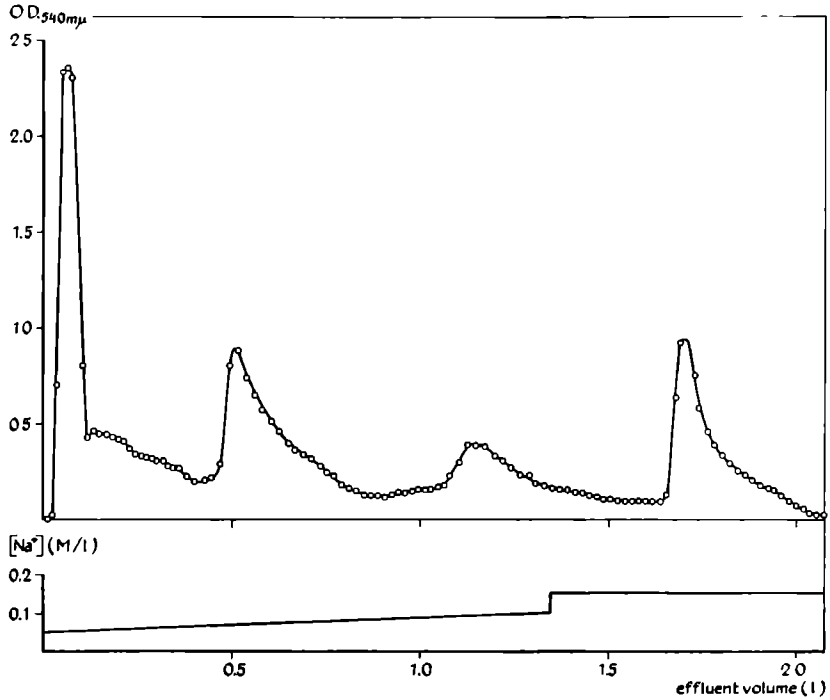


FIGURE 5.1 Elution pattern of bovine hemoglobin (2.5 g) treated with cyanate and applied to a Biorex 70 column (2 × 40 cm) which was equilibrated before with a 0.05 M sodium phosphate buffer (pH 6.2) at 4.0°C.

globin is shown in figure 5.1. Similar results were obtained when Amberlite CG 50 or Sephadex CM 50 were used. Biorex was preferred to Amberlite since preparation of this resin proceeds much faster, and to Sephadex because it showed no volume changes as occur in the gel bed at these low ionic strengths. The fractions obtained were concentrated on an Amincon diaflo apparatus and stored under CO at 4.0°C. Methemoglobin concentration of these solutions was about 8 % of the total hemoglobin concentration. The whole preparation procedure is summarized in table 5.1.

TABLE 5.1 Preparation of carbamylated derivatives of bovine carboxyhemoglobin.

Treatment	Concentration (moles/mole αβ-dimer)	pH	Temperature (°C)	Time (hour)
cystamine HCl	15	8.5	4	1
↓ GF*				
potassium cyanate	5	6.2	25	1
↓ GF				
dithiothreitol	15	8.5	4	12
↓ GF				
fractionation on Biorex-70		6.2	4	

* GF: gel filtration.

Identification of the four peaks obtained in fractionating the hemoglobin solution on Biorex was not carried out so far. Since the elution pattern was essentially the same as that obtained by Kilmartin and Rossi-Bernardi (1971), and titration studies performed on the automatic titration equipment of de Bruin *et al.* (1969) showed similar results as in the horse hemoglobin derivatives, fractions were assumed to correspond to those obtained for horse hemoglobin identified in the proper way.

2. *Determination of reactive sulfhydryl groups*

The efficiency of the blocking procedure of -SH groups with cystamine and their recovery after treatment with dithiothreitol were studied by spectrophotometric -SH determination with p-chloromercuribenzoate (PMB) according

to Boyer (1954). The procedure is based on the increase of optical density (O.D.) in the 250 mμ region accompanying mercaptide formation which varies linearly with the amount of sulfhydryl present in solution.
Spectrophotometric -SH determination was performed on solutions of CO hemoglobin in a phosphate buffer using a double beam spectrophotometer (Hitachi Perkin Elmer model 124). PMB was added to hemoglobin and reference solution by microburettes (Metrohm, type E 475). Stock solutions of PMB were standardized against glutathione. As reference solution a phosphate buffer was used to which $K_2Cr_2O_7$ was added. As shown in figure 5.2a $K_2Cr_2O_7$, in contrast to $K_3Fe(CN)_6$, does not react with PMB.

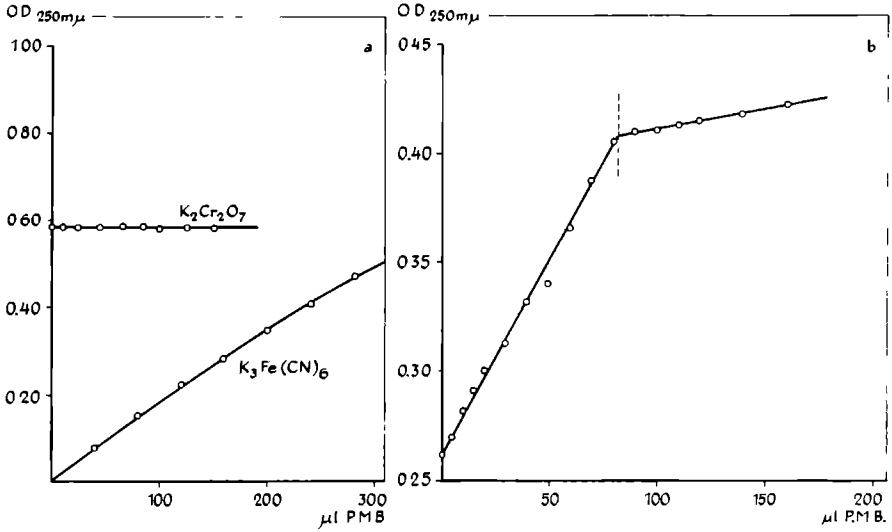


FIGURE 5.2
a Titration of $K_3Fe(CN)_6$ and $K_2Cr_2O_7$ with p-chloromercuribenzoate (PMB).
b Titration of bovine hemoglobin with p-chloromercuribenzoate (PMB).

A typical PMB titration curve of bovine hemoglobin at a pH of 7.0 is shown in figure 5.2b. The number of free -SH groups within the bovine CO hemoglobin tetramer obtained in this way was found to be 2.17 ± 0.13 (mean and standard deviation of three determinations). Blocking of these two -SH groups was studied with cystamine concentrations (15 and 50 moles per mole αβ-dimer) at two different pH values (7.1 and 8.5) and after four periods of reaction time ($\frac{1}{2}$,1,2 and 4 h). After treatment at 4°C the excess of cystamine was removed by gel filtration. Results are shown in table 5.2. In all cases both -SH groups

TABLE 5.2 Sulfhydryl titrations on bovine hemoglobin derivatives.

Treatment	Reaction conditions at 4.0°C: concentration (moles/mole αβ-dimer)	pH	time (hour)	Number of —SH groups titrated with PMB
cystamine HCl	15	7.1	4	none
	50	7.1	4	none
cystamine HCl	15	8.5	4	none
	50	8.5	4	none
cystamine HCl	15	7.1	½	none
	15	7.1	1	none
	15	7.1	2	none
dithiothreitol	15	7.1	4	2.31
	50	7.1	4	2.23
dithiothreitol	15	8.5	4	2.24
	50	8.5	4	2.14

of hemoglobin appear to be completely blocked. Deblocking of cystamine-blocked -SH groups by addition of dithiothreitol was studied at two levels of dithiothreitol concentration (15 and 50 moles/mole αβ-dimer) and at two different pH values (7.1 and 8.5). Excess of dithiothreitol was removed by gel filtration. It follows from table 5.2 that both -SH groups were completely recovered under these conditions.

3. *Oxygen equilibrium curves*

In determining the oxygen equilibrium curves of modified hemoglobins such as the cyanate-blocked derivatives, difficulties arise which are commonly absent when dealing with untreated hemoglobin. Usually modified hemoglobins are available only in small amounts or in very dilute solution. Besides, they are readily oxidized during the modification procedure (when not kept constantly under CO) or do so while oxygen equilibrium is studied. Due to shortage of material gasometric techniques cannot be applied in most cases. The spectrophotometric method commonly used, although consuming less material, does not account for methemoglobin formation during equilibration of hemoglobin with oxygen. Therefore, instead of following the two wave-length method used by most authors (Riggs, 1951), O.D. was measured at three different wavelengths, thus allowing to calculate methemoglobin concentration at any time during equilibration with oxygen.

a. Principle

The three wavelengths chosen were 523 mμ (which is an isobestic point of methemoglobin (MHb) and oxyhemoglobin (HbO_2)), 558 mμ (where a relatively large difference in absorbance exists between Hb, HbO_2 and MHb and the slope of the O.D. curve is not very steep), and finally 569 mμ (which is an isobestic point of Hb and HbO_2 whereas the difference of absorbance with MHb is relatively large). The O.D. values at these wavelengths are

$$O.D.^{523} = \varepsilon^{523}_{Hb}\,[Hb]l + \varepsilon^{523}_{HbO_2,MHb}\,([HbO_2] + [MHb])l \qquad (1)$$

$$O.D.^{558} = \varepsilon^{558}_{Hb}\,[Hb]l + \varepsilon^{558}_{HbO_2}\,[HbO_2]l + \varepsilon^{558}_{MHb}\,[MHb]l \qquad (2)$$

$$O.D.^{569} = \varepsilon^{569}_{Hb,HbO_2}\,([Hb] + [HbO_2])l + \varepsilon^{569}_{MHb}\,[MHb]l \qquad (3)$$

where ε is the millimolar extinction coefficient of the component, [Hb], [HbO_2] and [MHb] are the concentrations in mM/l, and l is the layer thickness of the absorption cuvette in cm.

ε values are estimated at each wavelength from hemoglobin solutions consisting of a single component. Once ε values are established oxygen saturation (S_{O_2}, %) and MHb are readily obtained from determination of O.D. at three wavelengths only.

b. Determination of ε values

ε values were determined on hemoglobin solutions prepared at 4°C from freshly drawn heparinized bovine blood. About 5 ml of whole blood were hemolyzed by addition of 100 ml bidistilled water, 0.5 ml Sterox S E was added and the final solution was diluted to one liter with the phosphate buffer. In the stock solution thus obtained hemoglobin concentration was about 45 μM (Fe)/l, phosphate concentration 0.02 M/l, ionic strength (after addition of KCl) 0.2 and pH 7.22. Equilibration of hemoglobin solutions was performed in a double-walled tonometer thermostated at 27.4°C and provided with an absorption cuvette joined to the tonometer. The cuvette could easily be disconnected for the purpose of cleaning. 10 ml of the stock solution were deoxygenated in the tonometer which was shaken on a linear shaker and flushed continuously with nitrogen preheated and humidified at the same temperature. After deoxygenation was complete, O.D. was measured at 522, 523, 524 mμ, at

558 mμ and at 568, 569 and 570 mμ. The hemoglobin solution was then oxygenated with 25 % oxygen in nitrogen and O.D. was determined at the same wavelengths. Finally the sample was oxidized by addition of 3.16 moles $K_3Fe(CN)_6$ per mole (Fe) hemoglobin and O.D. was measured at these wavelengths again. The calibration procedure was repeated for 10 hemoglobin samples. Standard error of the mean for O.D. readings of Hb, HbO_2 and MHb was 0.005 or less. The exact position of the isobestic points and their O.D. were derived graphically from O.D. readings of both components at adjacent wavelengths. After ε values were calculated equations (1), (2) and (3) were solved for [Hb], [HbO_2] and [MHb]. An example of a set of equations derived in this way is:

$$[MHb] = 0.0506\ (O.D.)_{522.7} - 0.0016\ (O.D.)_{588} - 0.0335\ (O.D.)_{569.5} \quad (4)$$

$$[HbO_2] = 0.1043\ (O.D.)_{522.7} - 0.0642\ (O.D.)_{588} - 2.1425\ [MHb] \quad (5)$$

$$[Hb] = 0.0307\ (O.D.)_{522.7} - 0.9837\ ([HbO_2] + [MHb]) \quad (6)$$

c. Determination of the oxygen equilibrium curve

The actual determination of the oxygen equilibrium curve was performed under conditions similar to those present during estimation of ε values. The hemoglobin solution was diluted in the same phosphate buffer to such extent that the O.D. reached was of the order of 0.4 (van Kampen and Zijlstra, 1965). Ten ml of this solution were deoxygenated and O.D. was read at the three wavelengths. This procedure was repeated after each equilibration of the sample with oxygen of varying concentrations. Three to five equilibrations could be performed on a single sample before a significant increase of [MHb] was noticed. Thus 5 μM (Fe) of hemoglobin are sufficient for an oxygen equilibrium curve of 12 points, including the preceding calibration procedure (in duplicate). Figure 5.3 shows a series of oxygen equilibrium curves of native (a,b,c,d) and double-blocked (e) hemoglobin in the presence of various hemoglobin concentrations (pH 7.40 and temperature 27.4°C). Carbon monoxide was removed from the modified derivative by equilibration with pure oxygen at 4°C under strong light. Replacement by oxygen was found to be complete (checked spectroscopically) within 20 min. Both Hill constant (n) and oxygen partial pressure at half saturation (P_{50}), calculated from the data between 10 and 90 % saturation, decreased at increasing methemoglobin concentration, in agreement with

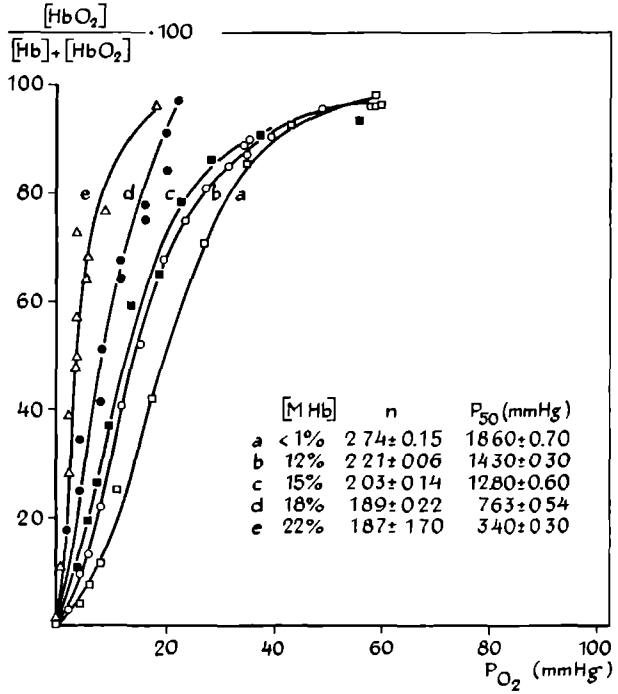


FIGURE 5.3 Oxygen equilibrium curves of untreated (a,b,c,d) and carbamylated double-blocked (e) bovine hemoglobin at varying methemoglobin concentration. pH 7.40 and temperature 27.4°C.

early and recent data (Darling and Roughton, 1942; Enoki *et al.*, 1969). It is concluded that the rather low n value of the double-blocked derivative is largely due to the high methemoglobin concentration present in solution.

DISCUSSION

Stark and Smyth (1963) and Stark (1964, 1965) showed that cyanate reacts, exept for terminal amino groups, with ε amino groups, -SH groups, tyrosines and histidines as well as with carboxyl groups. Under the conditions used the reactivity of the ε amino groups is very low. Reaction with tyrosine and histidine residues was reported to be reversible at alkaline conditions. Since the reaction with -SH groups, however, appeared to be not completely reversible in hemoglobin solutions at these conditions the reactive -SH group was protected by cystamine according to Taylor *et al.* (1966) before treatment with cyanate was

started. The reaction with carboxyl groups is reported to be irreversible, therefore further identification of the fractions obtained is needed before functional properties may be studied in detail. As concluded from titration curves, the first fraction showed the disappearance of one charge per heme which corresponds to the double-blocked derivatives of horse hemoglobin whereas the titration curve of the fourth fraction was identical to that of untreated bovine hemoglobin. Therefore it seems rather likely that the second and third fractions correspond to single-blocked derivatives carbamylated at the terminal amino group of either α or β chain.

As to its functional properties the presumably double-blocked derivative has been studied to some extent. It showed about normal heme-heme interaction although slightly reduced by the presence of large concentrations of methemoglobin, indicating that no large conformational change had occurred. Titration curves with nitrogen and oxygen alternately showed a slightly reduced Bohr effect, not influenced by the presence of CO_2. Carbamate estimation on this derivative showed no CO_2 binding at all. Functional properties of single-blocked derivatives will be studied in a similar way. The infrared method developed by Ortega *et al.* (1966) was modified in such a way as to make it suited for analysis of fluids of low CO_2 content. This method (presented in the Appendix) may be helpful in carbamate estimations of single-blocked derivatives.

Although a quantitative description of the mechanism of carbamate formation is not yet possible a few remarks may be made in relation to data presented in figure 4.8 which may elucidate the problem at least qualitatively. As already mentioned Pace *et al.* (1970) showed that the total amount of CO_2 bound to hemoglobin was reduced by about 25 % upon addition of 2,3-DPG whereas the total amount of CO_2 bound by oxyhemoglobin was not affected. Since under these conditions CO_2 will largely be displaced from its binding site on the β chain it follows that the α terminal amino group contributes for the greater part to oxylabile CO_2 binding and therefore $K_{\alpha Hb} \simeq 1.5\ 10^{-2}$ and $K_{\beta Hb} \simeq 0.46\ 10^{-2}$. The absence of any CO_2 effect of 2,3-DPG on bovine hemoglobin does not seem to invalidate these arguments. It presumably arises from the 'deleted' primary structure of the β chain without affecting its pK_z; no abnormally low pK_z values of terminal amino groups were observed hitherto in titration studies of bovine hemoglobin in comparison with pK_z values for human hemoglobin.

In the case of oxyhemoglobin the situation is less clear. The terminal amino

group of the β chain is shown to be not involved in the Bohr effect (Kilmartin and Rossi-Bernardi, 1969) which implies that its pK_z value remains essentially unchanged upon oxygenation. The terminal amino group of the α chain contributes significantly to the Bohr effect (25 %) by raising its pK_z upon deoxygenation (Perutz, 1970). Concerning CO_2 binding upon oxygenation there are two possibilities: either the affinity of the β terminal amino group is increased by a factor of about 2 and that of the α terminal amino group is reduced by a factor of 25, or both are lowered by a factor of 7 and 2 respectively.

As seen from equation (27) (chapter 4) $K_{c\beta}$ is expected to be raised by a factor of 2 in the first alternative and to be lowered by a factor of 7 in the second alternative. If $K_{z\alpha}$ is reduced by 1 pK unit when hemoglobin is oxygenated, $K_{c\alpha}$ would be lowered by a factor of about 250 in the first alternative and by a factor of about 20 in the second alternative. These factors are roughly 3 times smaller if $pK_{z\alpha}$ changes by 0.5 pK unit only.

In order to clarify this situation a model experiment was carried out. The terminal amino group of either α or β chain is reacted with CO_2 and the carbamate formed (shown in figure 5.4a) is considered with respect to its interactions with other residues within the protein molecule. Oxygen (O_1) is assumed to rotate freely around the axis of the $C_2 - N_3$ bond whereas C_2 is rotating at the

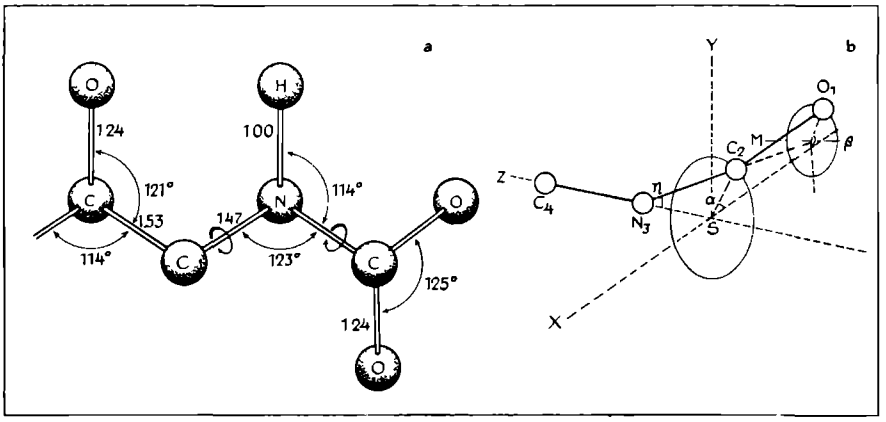


FIGURE 5.4

a Atomic model of a terminal amino group of hemoglobin reacted with CO_2. Bond lengths are indicated in Ångstrom.

b The same model located in a XYZ coordinate system. O_1 freely rotates around C_2M; C_2 rotates in the XY plane. Details are given in the text.

same time around the $N_3 - C_4$ axis in the XY plane (figure 5.4b). The coordinates of O_1 may be shown to obey

$$\vec{r}_{O1} = \begin{pmatrix} (a + d \sin \eta) \cos \alpha - b \cos \eta \cos \alpha \cos \beta + b \sin \alpha \sin \beta \\ (a + d \sin \eta) \sin \alpha - b \cos \eta \sin \alpha \cos \beta - b \cos \alpha \sin \beta \\ d \cos \eta + b \sin \eta \cos \beta \end{pmatrix}$$

where η refers to the (fixed) angle between C_2N_3 and N_3C_4, α to the angle between SC_2 and the XZ plane, and β to the angle between C_4SC_2 plane and O_1M; $C_2S = a$, $C_2M = d$ and $OM = b$.

Possible interactions of O_1 with other residues of hemoglobin may promote or impair CO_2 binding by affecting the K_c value of the terminal amino group to which it is attached. The range at which these interactions may occur is supposed to be within a distance of 3 Å from O_1. It may be shown that the shortest possible distance between O_1 and any residue is reached when X, O_1, C_2N_3 are all located in the same plane. From the coordinates of horse carboxyhemoglobin at a resolution of 2.8 Å (kindly provided by Dr. M. Perutz), computer calculation showed that no groups capable of interacting with O_1 were present at a distance of 3 Å or less. The group most adjacent to $O_{1\beta}$ was the terminal carboxyl group of its partner chain (minimum distance 3.45 Å) which will certainly reduce its $K_{c\beta}$ value (second alternative). The minimum distance between $O_{1\alpha}$ and the terminal carboxyl group of its partner chain was calculated to be 6.22 Å and will probably not influence $K_{c\alpha}$.

It is concluded that alterations of $K_{c\alpha,\beta}$ are mainly due to interactions of the carbamate group in hemoglobin rather than in oxyhemoglobin. Similar calculations may be performed on hemoglobin as soon as its coordinates are available (Bolton and Perutz, 1970).

The following arguments may be presented in favor of the second alternative. It gives an explanation of the decrease of $K_{c\beta}$ upon oxygenation whereas in first the alternative no indication for a rise of $K_{c\beta}$ is found. No evidence seems available to elucidate the drastic reduction of $K_{c\alpha}$ upon oxygenation in the first alternative whereas such a large decrease of $K_{c\alpha}$ does not occur in the second alternative. It therefore seems rather likely to suppose K_α to be $7.7\ 10^{-3}$ in oxyhemoglobin.

Terminal amino groups of both α and β chains are involved in oxylabile CO_2 binding but the contribution of the α terminal amino group is much more pronounced.

A preparation procedure of bovine hemoglobin derivatives carbamylated at their terminal amino groups is described as well as a method to study their oxygen equilibrium in the presence of high methemoglobin concentrations.
Heme-heme interaction of the double-blocked derivative was reduced ($n = 1.89$) and the curve was displaced to the left ($P_{50} = 3.4$ mmHg).
Oxygen equilibrium curves of untreated hemoglobin in the presence of about the same methemoglobin concentration showed similar n and P_{50} values. Carbamate formation is discussed in terms of ionization constants and CO_2 equilibrium constants of individual binding sites.

Estimation of low CO_2 content of fluids by infrared analysis

In order to perform carbamate determinations, also on single-blocked hemoglobin derivatives, accurate estimation of total CO_2 at low CO_2 content (0.05–0.5 mM/l) is required. Since at these concentrations the van Slyke technique is likely to produce errors ranging from 10 to 100 % a new method was developed with greater accuracy in the lower range of CO_2 content.

An infrared gas analyzer (URAS 2, Hartmann and Braun) was used with a cell of 250 mm length, corresponding to a full scale deflection of 0.01 % CO_2. The general set-up is shown in figure 5.5a, b. The inlet and outlet of the analyzer were connected to a gas extraction chamber provided with a sample inlet system. All connections were made of CO_2 impermeable viton tubing. The circulating gas flow of 120 ml/min was regulated by a membrane pump and a flowmeter.

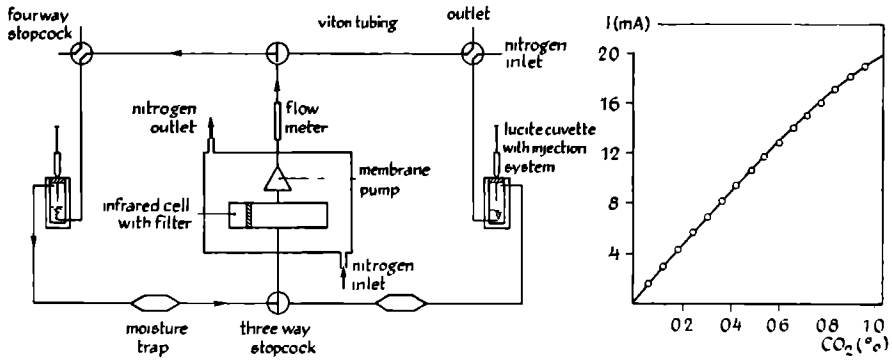


FIGURE 5.5 General set-up of the infrared unit for estimation of CO_2 content (left). Analyzer output (mA) for gases of varying CO_2 concentration (right).

In order to expel all CO_2 from the sample 3 ml of 0.1 N lactic acid with antifoam were added to the extraction chamber. A moisture trap was included in the circuit in order to prevent overflow of lactic acid into the cell.

Before the injection of the sample the circuit was flushed with CO_2 free nitrogen and then closed by turning the four-way stopcock. Time delay between subsequent determinations was diminished by adding a second circuit separated from the first one by two three-way stopcocks.

The analyzer was calibrated by mixing various amounts of air (containing 0.03 % CO_2) with CO_2 free nitrogen in a gas mixing pump (Wösthoff). In figure 5.5b the output of the gas analyzer is plotted against the CO_2 percentage of these gas mixtures. The calibration curve of the gas analyzer is not linear. At these low CO_2 concentrations water vapor in the closed circuit interferes with the CO_2 reading. This effect of cross-sensitivity could be eliminated completely by inserting a suitable filter supplied by the firm.

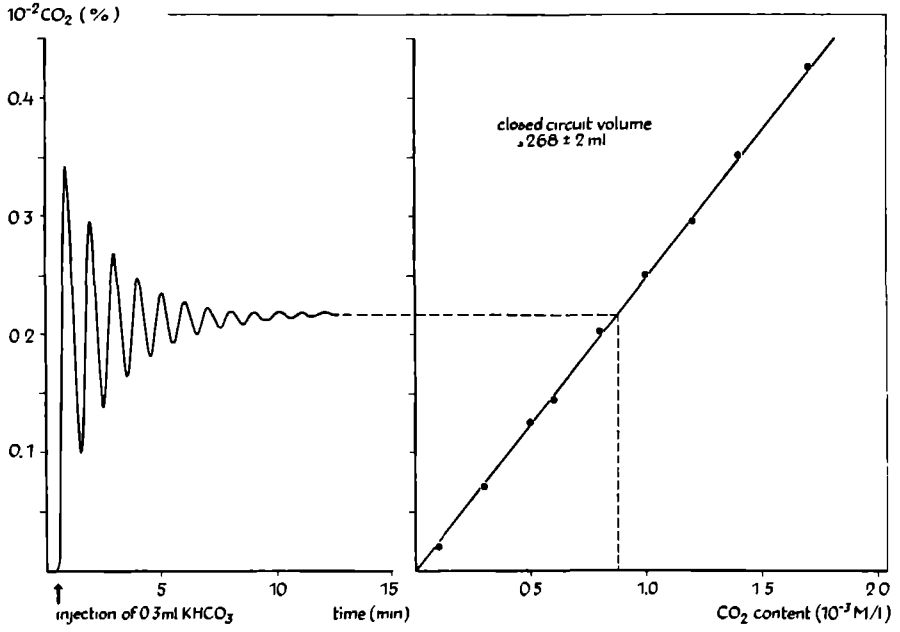


FIGURE 5.6 Time course of the analyzer output (% CO_2) after injection of 0.3 ml of 0.9 mM/l $KHCO_3$ into the closed circuit (left). Calibration line of CO_2 infrared gas analyzer with a closed circuit volume of 268 ± 2 ml (right).

Figure 5.6 shows the time course of the analyzer output after injection of 0.3 ml of a 0.9 millimolar solution of $KHCO_3$. The CO_2 content of this sample is readily obtained from a plot of the analyzer output in percentage of CO_2 against the CO_2 content of various solutions injected into the system. From similar experiments it is concluded that estimations of CO_2 content in the range of 0.1 mM/l are reproducible to within ± 0.003 mM/l (8 determinations, 0.5 ml sample volume.

CHAPTER I

BATES, R. G. (1954). *Electrometric pH Determinations.* New York, John Wiley and Sons, pp. 169–175.

BIEDERMANN, G. (1956). *Basicity constant of p-benzoquinone.* Acta Chem. Scand. **10**: 1340–1344.

BIILMANN, E. AND H. LUND (1921). *Sur l'électrode à quinhydrone.* Ann. Chim. **16**: 321–340.

BIILMANN, E. AND A. L. JENSEN (1927). *Sur le potentiel de l'électrode à quinhydrone par rapport à l'électrode à hydrogène.* Bull. Soc. Chim. **41**: 151–166.

BRAUDE, E. A. (1945). *Studies in light absorption. Part. I. p-Benzoquinones.* J. Chem. Soc.: 490–497.

CONSTANTINE, H. P., M. R. CRAW AND R. E. FORSTER (1965). *Rate of the reaction of carbon dioxide with human red blood cells.* Am. J. Physiol. **208**: 801–811.

DAHLGREN, G. AND M. J. GOODFRIEND (1970). *Quinhydrone electrode drift.* Anal. Chem. **42**: 111–113.

DINGEMANS, P. (1928). Thesis, Delft.

ELLER, W. AND K. KOCH (1920). *Synthetische Darstellung von Huminsäuren.* Ber. Deutsch. Chem. Gesell. **53**: 1469–1476.

GABBARD, J. L. (1947). *The effect of the hydrogen ion concentration upon the 'Salt error' of the quinhydrone electrode.* J. Am. Chem. Soc. **69**: 533–536.

GERTZ, K. H. AND H. H. LOESCHCKE (1958). *Elektrode zur Bestimmung des CO_2-Drucks.* Naturwissensch. **45**: 160–161.

HABER, F. AND R. RUSS (1904). *Ueber die elektrische Reduktion.* Z. physik. Chem. **47**: 257–335.

HARNED, H. S. AND D. D. WRIGHT (1933). *A study of the cell, Pt/quinhydrone, HCl (0.01)/AgCl/Ag, and the normal electrode potential of the quinhydrone electrode from 0 to 40.0° C.* J. Am. Chem. Soc. **55**: 4849–4857.

HOVORKA, F. AND W. C. DEARING (1935). *The 'Salt error' and normal electrode potential of the quinhydrone electrode at 25.0° C.* J. Am. Chem. Soc. **57**: 446–453.

JAMES, T. H., J. M. SNELL AND A. WEISSBERGER (1938). *Oxidation processes. XII. The autoxidation of hydroquinone and of the mono-, di- and trimethylhydroquinones.* J. Am. Chem. Soc. **60**: 2084–2093.

KIMMICH, H. P. AND F. KREUZER (1969). *Catheter P_{O_2} electrode with low flow dependency and fast response.* Progr. Resp. Res. vol. 3: 22–41. Basel, Karger.

LA MER, V. K. AND L. E. BAKER (1922). *The effect of substitution on the free energy of oxidation-reduction reactions.* J. Am. Chem. Soc. **44**: 1954–1964.

LA MER, V. K. AND E. K. RIDEAL (1924). *The influence of hydrogen concentration on the auto-oxidation of hydroquinone.* J. Am. Chem. Soc. **46**: 223–231.

LAMMERT, O. M., J. LIVINGSTON, R. MORGAN AND M. A. CAMPBELL (1931). *The quinhydrone electrode II.* J. Am. Chem. Soc. **53**: 597–604.

LEIGHTON, P. A. AND G. S. FORBES (1929). *The photochemical decomposition of benzoquinone in water and in alcohol.* J. Am. Chem. Soc. **51**: 3549–3560.

LINDERSTROM-LANG, K. (1927). *On the relation between the sizes of ions and the salting-out of hydroquinone and quinone.* C. R. trav. lab. Carlsberg **17**: 1–6.

LIVINGSTON, J., R. MORGAN, O. M. LAMMERT AND M. A. CAMPBELL (1932). *The preparation and reproducibility of the quinhydrone electrode.* Trans. Electrochem. Soc. **61**: 405–426.

LUNN, J. L. AND W. W. MAPLESON (1963). *The Severinghaus P_{CO_2} electrode: a theoretical and experimental assessment.* Brit. J. Anaesth. **35**: 666–678.

MASON, H. A. (1949). *The chemistry of melanin.* J. Biol. Chem. **181**: 803–808.

MICHAELIS, L. (1935). *Semiquinones, the intermediate steps of reversible organic oxidation-reduction.* Chem. Rev. **16**: 243–286.

MICHAELIS, L. AND S. GRANICK (1944). *Molecular compounds of the quinhydrone type in solution.* J. Am. Chem. Soc. **66**: 1023–1029.

ROSENTHAL, R., A. E. LORCH AND L. P. HAMMETT (1937). *The kinetics of the quinhydrone electrode reaction.* J. Am. Chem. Soc. **56**: 1795–1804.

RYBAK, B. AND H. PENFORNIS (1969). *Micro-sondes cathétérisables pour pH et P_{CO_2}.* J. Physiol. (Paris) **61**: 394.

SCHEIBE, G. (1926). *Die Veränderlichkeit der Absorptionsspektren in Lösungen und die Ladungsverteilung der Moleküle.* Ber. Deutsch. Chem. Gesell. **59**: 2617–2628.

SCHULER, R. AND F. KREUZER (1967). *Rapid polarographic in vivo oxygen catheter electrodes.* Respir. Physiol. **3**: 90–110.

SEVERINGHAUS, J. W. AND A. F. BRADLEY (1958). *Electrodes for blood P_{O_2} and P_{CO_2} determination.* J. Appl. Physiol. **13**: 515–520.

SORENSEN, S. P. L., M. SORENSEN AND K. LINDERSTROM-LANG (1923). *Sur l' 'erreur de sel' inhérente à l'électrode de quinhydrone.* Compt. rend. trav. lab. Carlsberg **14**: 1–31.

STOW, R. W. AND B. F. RANDALL (1954). *Electrical measurement of the P_{CO_2} of blood.* Am. J. Physiol. **179**: 678.

STOW, R. W., R. F. BAER AND B. F. RANDALL (1957). *Rapid measurements of the tension of carbon dioxide in blood.* Arch. Phys. Med. Rehab. **38**: 646–650.

VEIBEL, S. (1923). *The quinhydrone electrode as a comparison electrode.* J. Chem. Soc. **123**: 2203–2207.

WAGNER, C. AND K. GRÜNEWALD (1940). *Untersuchungen über die Molekülarten in wässrigen Chinhydron-Lösungen.* Z. Elektrochem. **46**: 265–269.

WEAST, R. C. AND S. M. SELBY EDS. (1966). *Handbook of Chemistry and Physics, 47th edition.* Cleveland, Ohio, The Chemical Rubber Co.

CHAPTER 2

ALT, H., H. BINDER, A. KÖHLING AND G. SANDSTEDE (1971). *Redox-Verhalten unlöslicher Chinone/Hydrochinone.* Angew. Chem. **83**: 502–504.

BENEKEN KOLMER, H. H. AND F. KREUZER (1968). *Continuous polarographic recording of oxygen pressure in respiratory air.* Respir. Physiol. **4**: 109–117.

KIMMICH, H. P. AND F. KREUZER (1967). *Telemetry of respiratory oxygen pressure in man during exercise.* Digest 7th Internat. Conf. Med. Biol. Engin., Stockholm. p. 90.

KIMMICH, H. P. AND F. KREUZER (1968). *Catheter PO_2 electrode with low flow dependency and fast response.* Int. Symposium on Oxygen Pressure Recording, Nijmegen. Prog. Resp. Res., vol. 3: 100–110. Basel, Karger.

LAITINEN, H. A. AND I. M. KOLTHOFF (1941). *Voltammetry with stationary microelectrodes of platinum wire.* J. Phys. Chem. **45**: 1061–1079.

LIVINGSTON, J., R. MORGAN, O. M. LAMMERT AND M. A. CAMPBELL (1931). *The quinhydrone electrode.* I. J. Am. Chem. Soc. **53**: 454–469.

MÜLLER, O. H. (1940). *Oxidation-Reduction potentials measured with the dropping mercury electrode. III. Polarographic study of quinhydrone in buffered and unbuffered solutions.* J. Am. Chem. Soc. **62**: 2434–2441.

MÜLLER, O. H. (1947). *Polarographic study with a microelectrode past which an electrolyte is flowing.* J. Am. Chem. Soc. **69**: 2992–2997.

SCHULER, R. AND F. KREUZER (1967). *Rapid polarographic in vivo oxygen catheter electrodes.* Respir. Physiol. **3**: 90–110.

SEVERINGHAUS, J. W. AND A. F. BRADLEY (1958). *Electrodes for blood P_{O_2} and P_{CO_2} determination.* J. Appl. Physiol. **13**: 513–520.

VETTER, K. J. (1952). *Überspannung und Kinetik der Chinhydronelektrode.* Z. Elektrochem. **56**: 797–806.

CHAPTER 3

BRUGGEN, VAN J. T. AND J. C. SCOTT (1962). *Microdetermination of carbon dioxide.* Anal. Biochem. **3**: 464–471.

EIGEN, M., K. KUSTIN AND G. MAASS (1961). *Die Geschwindigkeit der Hydratation von SO_2 in wässriger Lösung.* Z. Physik. Chem. (N.F.) **30**: 130–136.

GIBBONS, B. H. AND J. T. EDSALL (1963). *Rate of hydration of carbon dioxide and dehydration of carbonic acid at 25.0° C.* J. Biol. Chem. **238**: 3502–3507.

HARNED, H. S. AND R. DAVIS, JR. (1943). *The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50.0° C.* J. Am. Chem. Soc. **65**: 2030–2037.

HARNED, H. S. AND S. R. SCHOLES, JR. (1941). *The ionization constant of HCO_3^- from 0 to 50.0° C.* J. Am. Chem. Soc. **63**: 1706–1709.

JONES, G. AND B. C. BRADSHAW (1933). *The measurement of the conductance of electrolytes. V. A redetermination of the conductance of standard potassium chloride solutions in absolute units.* J. Am. Chem. Soc. **55**: 1780–1800.

JONES, G. AND S. M. CHRISTIAN (1935). *The measurement of the conductance of electrolytes. VI. Galvanic polarization by alternating current.* J. Am. Chem. Soc. **57**: 272–280.

KEMPEN, VAN L. H. J., H. DEURENBERG AND F. KREUZER (1972). *The CO_2-quinhydrone electrode. A new method to measure partial CO_2 pressure in gases and liquids.* Respir. Physiol. **14**: 366–381.

LUNN, J. N. AND W. W. MAPLESON (1963). *The Severinghaus P_{CO_2} electrode; a theoretical and experimental assessment.* Brit. J. Anaesth, **35**: 666–678.

MAFFLY, R. H. (1968). *A conductometric method for measuring micromolar quantities of carbon dioxide.* Anal. Biochem. **23**: 252–262.

MURAKAMI, I., S. TAKASHIMA, F. KUJISAKU, H. SASAMOTO, Y. TAKAGI, Y. OOTA (1965). *A new method for determination of P_{CO_2} both in liquid and gas.* Digest 6th Internat. Conf. Med. Electron. Biol. Engin., Tokyo. p. 610–611.

ROBINSON, R. A. AND R. H. STOKES (1959). *Electrolyte solutions.* London, Butterworths, pp. 87–99 and 465.

STOW, R. W., R. F. BAER AND B. F. RANDALL (1957). *Rapid measurements of the tension of carbon dioxide in blood.* Arch. Phys. Med. Rehab. **38**: 646–650.

TAYLOR, G. (1953). *Dispersion of soluble matter in solvent flowing slowly through a tube.* Proc. Roy. Soc. A **219**: 186–203.

TSAO, M. U. AND A. VADNEY (1964). *A method for continuous measurement of blood P_{O_2} and P_{CO_2}.* J. Lab. Clin. Med. **63**: 1041–1053.

ADAIR, G. S. AND M. E. ADAIR (1934). *The determination of the isoelectric and iso-ionic points of haemoglobin from measurements of membrane potentials.* Biochem. J. **28**: 1230–1258.

ALBERT, A. AND E. P. SERJEANT (1962). *Ionization constants of acids and bases.* London, Methuen, p. 24.

BAILEY, J. E., J. G. BEETLESTONE AND D. H. IRVINE (1970). *Reactivity differences between haemoglobins.* XVII. *The variability of the Bohr effect between species and the effect of 2,3-Diphosphoglyceric acid on the Bohr effect.* J. Chem. Soc. (A) **5**: 756–763.

BAUER, CH. (1970). *Reduction of the carbon dioxide affinity of human haemoglobin solutions by 2,3 diphosphoglycerate.* Respir. Physiol. **10**: 10–19.

BENESCH, R. AND R. E. BENESCH (1967). *The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin.* Biochem. Biophys. Res. Comm. **26**: 162–167.

BRINKMAN, R. AND J. H. P. JONXIS (1936). *Alkaline resistance and spreading velocity of foetal and adult types of mammalian haemoglobin.* J. Physiol. **88**: 162–166.

BUNN, H. F. AND R. W. BRIEHL (1970). *The interaction of 2,3 diphosphoglycerate with various human hemoglobins.* J. Clin. Invest. **49**: 1088–1095.

CHANUTIN, A. AND R. R. CURNISH (1967). *Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes.* Arch. Biochem. Biophys. **121**: 97–102.

CHIPPERFIELD, J. R. (1966). *The kinetics of combination of carbon dioxide with the anions of glycine, glycyl-glycine, and related amino acids.* Proc. Roy. Soc. B **164**: 401–410.

DAVIES, C. W. AND G. H. NANCOLLAS (1950). *Preparation of carbonate-free sodium hydroxide.* Nature **165**: 237–238.

DAYHOFF, M. O. (1969). *Atlas of protein sequence and structure.* Nat. Biochem. Res. Foundation, vol. 4, Silver Spring, p. D 61–62.

EIGEN, M. AND L. DE MAEYER (1963). *Technique of organic chemistry.* Vol. 8, Ed. 2, Interscience Publishers, New York, Chapter XVIII, p. 1034.

FALBE-HANSEN, I. (1961). *Spectrophotometric studies on denatured globin hemichrome.* Scand. J. Clin. Lab. Invest. **13**: 429–433.

FAURHOLT, C. (1925). *Etudes sur les solutions aqueuses de carbamates et de carbonates.* J. Chim. Phys. **22**: 1–44.

FERGUSON, J. K. W. (1937). *Carbamino compounds of CO_2 with human haemoglobin and their role in the transport of CO_2.* J. Physiol. **88**: 40–55.

FERGUSON, J. K. W. AND F. J. W. ROUGHTON (1934). *The direct chemical estimation of carbamino compounds of CO_2 with haemoglobin.* J. Physiol. **83**: 68–86.

FERGUSON, J. K. W. AND F. J. W. ROUGHTON (1934). *The chemical relationships and physiological importance of carbamino compounds of CO_2 with haemoglobin.* J. Physiol. **83**: 87–102.

HANLON, S., G. NAGEL, R. BOYD, R. LEWSHENIA, M. FORSBERG AND S. CHEUNG (1971). *The dissociation of bovine hemoglobin at acid pH.* Biochim. Biophys. Acta **229**: 359–367.

HARNED, H. S. AND R. DAVIS, JR. (1943). *The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0 to 50.0° C.* J. Am. Chem. Soc. **65**: 2030–2037.

HARNED, H. S. AND S. R. SCHOLES, JR. (1941). *The ionization constant of HCO_3^- from 0 to 50.0° C.* J. Am. Chem. Soc. **63**: 1706–1709.

HASTINGS, A. B. AND J. SENDROY (1925). *The effect of variation of ionic strength on the apparent first and second dissociation constants of carbonic acid.* J. Biol. Chem. **65**: 445–455.

HAUROWITZ, F., R. L. HARDIN AND M. DICKS (1954). *Denaturation of hemoglobins by alkali.* J. Am. Chem. Soc. **58**: 103–105.

HENRIQUES, O. M. (1928). *Die Bindungsweise des Kohlendioxyds im Blute.* Biochem. Z. **200**: 1–24.

HERMANN, H., B. HUDOFFSKY, H. NETTER UND L. TRAVIA (1939). *Ueber den spezifischen Einfluss der Kohlensäure auf die Sauerstoffbindungskurve des Hämoglobins.* Pflügers Arch. ges. Physiol. **242**: 311–327.

International Committee for Standardization in Haematology, *Recommendations for haemoglobinometry in human blood.* Br. J. Haemat. **13**: Suppl. 71, 1967.

KAMPEN, VAN, E. J. AND W. G. ZIJLSTRA (1961). *Standardization of hemoglobinometry.* II. *The hemiglobincyanide method.* Clin. Chim. Acta **6**: 538–544.

KAPLAN, J. C. (1965). *Méthode de mesure rapide du taux de la methémoglobine dans les globules rouges.* Rev. Franç. Etudes Clin. et Biol. **10**: 856–859.

KEMPEN, VAN L. H. J. AND F. KREUZER (1971). *Carbamate equilibrium of bovine hemoglobin at 37.0° C.* In: Alfred Benzon Symposium IV: Oxygen affinity of hemoglobin and red cell acid-base status, Copenhagen, 17—22 May, 1971 (in press).

KILMARTIN, J. V. AND L. ROSSI-BERNARDI (1969). *Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups.* Nature **222**: 1243–1246.

KREUZER, F., F. J. W. ROUGHTON, L. ROSSI-BERNARDI AND J. KERNOHAN (1971). *Specific effect of CO_2 and bicarbonate on the affinity of hemoglobin for oxygen.* In: Alfred Benzon Symposium IV: Oxygen affinity of hemoglobin and red cell acid-base status, Copenhagen, 17–22 May, *1971* (in press).

LEMBERG, R. AND J. W. LEGGE (1949). *Haematin compounds and bile pigments. Their constitution, metabolism and function.* Interscience, New York, p. 228.

MARGARIA, R. (1957). *The contribution of hemoglobin to acid-base equilibrium of the blood in health and disease.* Clin. Chem. **3**: 306–318.

MILLA, E., G. GIUSTINA AND R. MARGARIA (1953). *L'esistenza di due tipi di composti dell' Hb col CO_2.* Giornale di Biochimica **2**: 153–165.

PACE, M., L. ROSSI-BERNARDI AND F. J. W. ROUGHTON (1970). *The effect of organic phosphates on the reactions of haemoglobin and oxyhaemoglobin with carbon dioxide.* Biochem. J. **119**: 67–68 P.

PERRELLA, M., M. PACE, P. JEAN, L. ROSSI-BERNARDI AND F. J. W. ROUGHTON (1971). *The carbamate equilibrium of bovine hemoglobin at 25.0° C.* In: Alfred Benzon Symposium IV: Oxygen affinity of hemoglobin and red cell acid-base status, Copenhagen, 17–22 May, *1971* (in press).

PERUTZ, M. F. (1970). *Stereochemistry of cooperative effects in haemoglobin.* Nature **228**: 726–739.

PINSENT, B. R. W., L. PEARSON AND F. J. W. ROUGHTON (1956). *The kinetics of combination of carbon dioxide with hydroxide ions.* Trans. Faraday Soc. **52**: 1512–1520.

RAPOPORT, S. (1969). *Regulation of concentration of DPG and ATP in red blood cells.* Försvarsmedicin **5**: 168–174.

RIEDER, R. F. (1970). *Hemoglobin stability: Observations on the denaturation of normal and abnormal hemoglobins by oxidant dyes, heat and alkali.* J. Clin. Invest. **49**: 2369–2376.

RISPENS, P., C. W. DELLEBARRE, D. ELEVELD, W. HELDER AND W. G. ZIJLSTRA (1968). *The apparent first dissociation constant of carbonic acid in plasma between 16 and 42.5° C.* Clin. Chim. Acta **22**: 627–637.

ROSSI-BERNARDI, L., M. PACE, F. J. W. ROUGHTON AND L. H. J. VAN KEMPEN (1969). *The estimation of hemoglobin-CO_2 compounds by gel filtration and ion-exchange chromatography.* In: CO_2: Chemical, Biochemical and Physiological Aspects. Eds. R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton. NASA no. SP–188. Washington: U.S. Government Printing Office, p. 65–71.

ROSSI-BERNARDI, L. AND F. J. W. ROUGHTON (1967). *The specific influence of carbon dioxide an carbamate compounds on the buffer power and Bohr effects in human haemoglobin solutions.* J. Physiol. **189**: 1–29.

ROUGHTON, F. J. W. (1964). *Handbook of Physiology*, Section 3: Respiration, vol. I. Eds. W. O. Fenn and H. Rahn, Washington: American Physiological Society. p. 767–825.

ROUGHTON, F. J. W. (1970). *Some recent work on the interactions of oxygen, carbon dioxide and haemoglobin.* Biochem. J. **117**: 801–812.

ROUGHTON, F. J. W. AND L. ROSSI-BERNARDI (1969). *Studies on the pK and rate of dissociation of the glycylglycine-carbamic acid molecule.* In: CO_2: Chemical, Biochemical and Physiological Aspects. Eds. R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton. NASA nr. SP–188. Washington: U.S. Government Printing Office, p. 41–45.

SCHROEDER, W. A., J. R. SHELTON, J. B. SHELTON, B. ROBBERSON AND D. R. BABIN (1967). *A comparison of amino acid sequences in the β-chains of adult bovine hemoglobins A and B.* Arch. Biochem. Biophys. **120**: 124–135.

SIDWELL, JR. A. E., R. H. MUNCH, E. S. GUZMAN BARRON AND T. R. HOGNESS (1938). *The salt effect on the hemoglobin-oxygen equilibrium.* J. Biol. Chem. **123**: 335–350.

SIGGAARD-ANDERSEN, O. (1962). *The first dissociation exponent of carbonic acid as a function of pH.* Scandinav. J. Clin. Lab. Invest. **14**: 587–597.

STADIE, W. C. AND H. O'BRIEN (1937). *The carbamate equilibrium.* II. *The equilibrium of oxyhemoglobin and reduced hemoglobin.* J. Biol. Chem. **117**: 439–470.

STADIE, W. C. AND E. R. HAWES (1928). *Studies on the oxygen-, acid-, and base-combining properties of blood.* J. Biol. Chem. **77**: 265–301.

TOMITA, S. AND A. RIGGS (1971). *Studies of the interaction of 2,3-diphosphoglycerate and carbon dioxide with hemoglobins from mouse, man and elephant.* J. Biol. Chem. **246**: 547–554.

ZIJLSTRA, W. G., E. J. VAN KAMPEN AND O. W. VAN ASSENDELFT (1969). *Standardization of haemoglobinometry. Establishing the reference point.* Proc. Kon. Ned. Akad. Wet., series C, **72**: 231–237.

CHAPTER 5

ADAIR, G. S. AND M. E. ADAIR (1934). *The determination of the isoelectric and iso-ionic points of haemoglobin from measurements of membrane potentials.* Biochem. J. **28**: 1230–1258.

BOLTON, W. AND M. F. PERUTZ (1970). *Three dimensional Fourier synthesis of horse deoxyhaemoglobin at 2.8 Å resolution.* Nature **228**: 551–552.

BOYER, P. D. (1954). *Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials.* J. Am. Chem. Soc. **76**: 4331–4337.

BRUIN, DE, S. H., L. H. M. JANSSEN AND G. A. J. VAN OS (1969). *Study of the Bohr groups of bovine hemoglobin.* Biochim. Biophys. Acta **188**: 207–215.

BUNN, H. F. AND R. W. BRIEHL (1970). *The interaction of 2,3-diphosphoglycerate with various human hemoglobins.* J. Clin. Invest. **49**: 1088–1095.

DARLING, R. C. AND F. J. W. ROUGHTON (1942). *The effect of methemoglobin on the equilibrium between oxygen and hemoglobin.* Am. J. Physiol. **137**: 56–68.

ENOKI, Y., H. TOKUI AND I. TYUMA (1969). *Oxygen equilibria of partially oxidized hemoglobin.* Respir. Physiol. **7**: 300–309.

HILL, R. J. AND R. W. DAVIS (1967). *The pK of specific groups of proteins.* I. *The α-amino group of the α chain of human CO-hemoglobin.* J. Biol. Chem. **242**: 2005–2013.

KAMPEN, VAN E. J. AND W. G. ZIJLSTRA (1965). *Determination of hemoglobin and its derivatives.* Advan. Clin. Chem. **8**: 141–186.

KILMARTIN, J. V. AND L. ROSSI-BERNARDI (1969). *Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups.* Nature, **222**: 1243–1246.

KILMARTIN, J. V. AND L. ROSSI-BERNARDI (1971). *The binding of carbon dioxide by horse haemoglobin.* Biochem. J. **124**: 31–45.

NEER, E. J. AND W. KONIGSBERG (1968). *The characterization of modified human hemoglobin.* II. *Reaction with 1-fluoro-2, 4-dinitrobenzene.* J. Biol. Chem. **243**: 1966–1970.

ORTEGA, F. G., S. A. M. ORIE AND G. J. TAMMELING (1966). *Determination of carbon dioxide content of blood by infrared analysis.* J. Appl. Physiol. **21**: 1377–1380.

PACE, M., L. ROSSI-BERNARDI AND F. J. W. ROUGHTON (1970). *The effect of organic phosphates on the reactions of haemoglobin and oxyhaemoglobin (carboxyhaemoglobin) with carbon dioxide.* Biochem. J. **119**: 67–68 P.

PERUTZ, M. F., H. MUIRHEAD, L. MAZZARELLA, R. A. CROWTHER, J. GREER AND J. V. KILMARTIN (1969). *Identification of residues responsible for the alkaline Bohr effect in haemoglobin.* Nature **222**: 1240–1243.

PERUTZ, M. F. (1970). *Stereochemistry of cooperative effects in haemoglobin.* Nature **228**: 726–739.

RIGGS, A. (1951). *The metamorphosis of hemoglobin in the bullfrog.* J. Gen. Physiol. **35**: 23–40.

STARK, G. R. AND D. G. SMYTH (1963). *The use of cyanate for the determination of NH_2-terminal residues in proteins.* J. Biol. Chem. **238**: 214–230.

STARK, G. R. (1964). *On the reversible reaction of cyanate with sulfhydryl groups and the determination of NH_2-terminal cysteine and cystine in proteins.* J. Biol. Chem. **239**: 1411–1414.

STARK, G. R. (1965). *Reactions of cyanate with functional groups of proteins.* III. *Reactions with amino and carboxyl groups.* Biochem. **4**: 1030–1036.

TAYLOR, J. F., E. ANTONINI, M. BRUNORI AND J. WYMAN (1966). *Studies on human hemoglobin treated with various sulfhydryl reagents.* J. Biol. Chem. **241**: 241–248.

STELLINGEN

I

Carbamino-hemoglobine kan beschouwd worden als een in vivo modificatie van hemoglobine, waarbij de configuratie van het hemoglobine molecuul een zo geringe wijziging ondergaat dat de 'heme-heme' interactie volledig behouden blijft.

2

Onder fysiologische omstandigheden treedt CO_2 uitsluitend in interactie met terminale amino groepen van de α en ß ketens van hemoglobine; dit geldt zowel voor hemoglobine in niet geligandeerde toestand als voor oxyhemoglobine.

3

Het 'oxylabiele' karakter van de CO_2-hemoglobine binding berust voornamelijk op een wijziging van de carbamino evenwichtsconstante tijdens oxygenering van hemoglobine. Terminale aminogroepen van α en ß ketens dragen hieraan bij, echter niet in gelijke mate.

4

De afwezigheid van enig meetbaar effect van 2,3-diphosphoglyceraat op de CO_2 bindende eigenschappen van runder hemoglobine kan op grond van de primaire structuur van zijn ß keten aannemelijk worden gemaakt.

5

De continue registratie van de partiele CO_2 spanning in vivo met behulp van catheter electroden, gebaseerd op redox potentiaal- of geleidbaarheidsmeting opent nieuwe perspectieven voor de klinische en experimentele geneeskunde.

6

Meting van de CO_2 afgifte door de huid met behulp van de infrarood gas analyse techniek volgens van Kempen en Thiele verdient de voorkeur boven de eerder toegepaste alkali-neutralisatie methode.

7

De hoge mate van CO_2 afgifte door de huid waargenomen bij patiënten met gegeneraliseerde erythrodermieën wettigt de veronderstelling dat bij deze patiënten meetbare verschuivingen in het zuur-base evenwicht optreden.

8

De invoering van het genormaliseerde eenheden stelsel in de klinische chemie leidt, afgezien van de evidente voordelen welke het biedt, tot een aantal inconsequenties die in het voorheen toegepaste niet gestandaardiseerde systeem afwezig waren.

9

Het is wenselijk dat fabrikanten van vaatwasmiddelen de samenstelling van toegevoegde producten-verbeteraars (additives) aan hun afnemers bekend maken.

10

De voortdurende inflatie van de gulden komt in het aandelenkapitaal van het merendeel der nederlandse ondernemingen niet of onvoldoende tot uitdrukking. De winst en de op basis hiervan vastgestelde dividenden geven derhalve een veel te rooskleurig beeld van de feitelijke rentabiliteit van het geinvesteerd vermogen.

11

Het recente 'intercity' project van de nederlandse spoorwegen ten spijt lijkt de verbinding Roermond–Utrecht aanmerkelijk slechter dan Roermond–Rome.

8 juni 1972

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